# Regulation of the Intestinal Bile Acid Transporter Genes by the Transcription Factors CDX1 and CDX2

---

**Dissertation**

**zur**

**Erlangung der naturwissenschaftlichen Doktorwürde**
**(Dr. sc. nat.)**

**vorgelegt der**

**Mathematisch-naturwissenschaftlichen Fakultät**

**der**

**Universität Zürich**

**von**

Li Ma

**aus**

China

**Promotionskomitee**

Prof. Dr. Carsten Wagner (Verantwortliches Fakultätsmitglied)

Prof. Dr. Gerd Kullak-Ublick (Leitung der Dissertation)

PD. Dr. Jyrki Eloranta

Prof. Dr. Béatrice Desvergne

**Zürich 2012**

# Dedication

To my Father Lian-Sheng Ma

and Mother Diao-Mei Guo

# SUMMARY

Membrane transport proteins carry nutrients and other compounds into the cell and eliminate toxic metabolites and xenobiotic agents in order to maintain cellular homeostasis. These transporters are designed to facilitate the transport of bile acids, peptides, amino acids, fatty acids, and other compounds. Protein malfunction or genetic polymorphisms can influence the transport activity of these proteins.

The apical sodium-dependent bile acid transporter (ASBT; gene symbol *SLC10A2*) is expressed abundantly in the ileum and mediates bile acid (BA) absorption across apical membranes of ileocytes. Caudal-type homeobox proteins CDX1 and CDX2 are transcription factors that regulate genes involved in intestinal epithelial differentiation and proliferation. The first aim of my research was to study the aberrant expression of both ASBT and CDXs in Barrett's esophagus (BE), and whether expression of the *ASBT* gene was regulated by CDXs. Short interfering RNA-mediated knockdown of CDXs resulted in reduced ASBT mRNA expression in intestinal cells. CDXs strongly induced the activity of the *ASBT* promoter in reporter assays in esophageal and intestinal cells. Nine CDX binding sites were predicted *in silico* within the *ASBT* promoter, and binding of CDXs to six of them was verified *in vitro* and within living cells by electrophoretic mobility shift assays and chromatin immunoprecipitation assays, respectively. RNAs were extracted from esophageal biopsies from 20 BE patients, and analyzed by real-time PCR. Correlation with ASBT mRNA expression was found for CDX1, CDX2, and hepatocyte nuclear factor-1α (HNF-1α) mRNA in BE biopsies. The human *ASBT* promoter was activated transcriptionally by CDX1 and CDX2. The results provide a possible explanation for the reported observation that ASBT is aberrantly expressed in esophageal metaplasia that also expresses CDX transcription factors. I have also presented preliminary results on organic solute transporter (*OST*) *α/β* promoter regulation by CDXs, whereby silencing CDX1 and CDX2 expression reduced endogenous OSTα/β at the mRNA level, and overexpression of CDXs elevated *OSTα* promoter activity.

The role of CDX factors in intestinal differentiation and their aberrant expression levels in intestinal metaplasia are well established. However, relatively little is known about the exact molecular mechanisms by which CDXs exert their function as transcriptional

activators or repressors. The second aim of my research was to identify protein interacting partners of CDXs that may mediate or modulate their transcriptional activity. To this end, I constructed a phage cDNA expression library using poly(A) RNA derived from the human small intestine and colon. Characterization of the library revealed it to be of high quality. The unamplified library contained $4.5\times10^6$ independent clones, with 99.6% of the phage vectors containing an insert. I performed primary far western screening ($1.7\times10^6$ clones screened per CDX protein) and secondary protein interaction screening using $^{35}$S-radiolabelled *in vitro* translated CDX1 and CDX2 proteins. The screening identified several clones encoding potential novel molecules that interact with CDX. One promising cDNA clone encoded the BRD1 protein, which contains a bromodomain that is frequently present in transcriptional coactivator proteins. I am currently at the validation phase to confirm that the positive clones from the far western screening encode true interacting partners.

# ZUSAMMENFASSUNG

Membrantransportproteine transportieren Nährstoffe in, sowie toxische Abbauprodukte und Xenobiotika aus der Zelle hinaus. Wichtige Substrate von Transportproteinen umfassen wichtige Verbindungen wie z.B. Peptide, Aminosäuren oder Fettsäuren. Proteinfehlfunktionen oder genetische Polymorphismen können die Transportaktivität dieser Transportproteine beeinflussen.

Der Apical sodium-dependent bile acid transporter (ASBT) wird vor allem im Ileum exprimiert und ist massgeblich an der Gallensalzaufnahme über die apikale Membran beteiligt. Caudal-type homeobox proteins CDX1 and CDX2 sind Transkriptionsfaktoren, welche Gene regulieren, die eine Rolle in der Differenzierung und Proliferation von intestinalen Epithelzellen spielen. Das erste Ziel meiner Forschungsarbeit war es, zu untersuchen, ob Abweichungen der ASBT- und CDX- Expression in Barrett-Ösophagus-Gewebe (BE-Gewebe) nachzuweisen sind und zu ergründen, ob die Expression des ASBT-Genes von CDX reguliert wird. Mittels si-RNA-knockdown-Experimenten in intestinalen Zelllinien, welche die CDX-Expression herabsetzten, konnte gezeigt werden, dass die ASBT-Expression, quantifiziert mittels ASBT-mRNA-Konzentrationsbestimmung, signifikant herabgesetzt wurde. In Gen-Reporter-Assays, durchgeführt in ösophagialen und intestinalen Zelllinien, konnte gezeigt werden, dass CDXs die Fähigkeit haben, den ASBT-Promoter stark zu induzieren. Neun CDX-Bindungsstellen wurden durch *in-silico*-Analyse in der ASBT-Promotorregion identifiziert. Die Bindung von CDXs an sechs der neun vorhergesagten Bindungsstellen konnte *in vitro* mittels Elektromobilität-Shift-Analyse (EMSA) und in intakten Zellen mittels Chromatin-Immunopräzipitation bestätigt werden. Die aus Biopsien von 20 BE-Patienten extrahierte RNA wurde mittels Realtime-PCR analysiert. Hier konnte eine Korrelation zwischen der Expression von ASBT und CDX1, CDX2 sowie dem hepatocyte nuclear factor-1α (HNF1α)nachgewiesen werden. Der Humane ASBT-Promoter wird auf Transkriptionsebene durch CDX1 und CDX2 aktiviert. Dieses Resultat könnte eine mögliche Erklärung für die zuvor beobachtete aberrante Expression von ASBT in metaplastischem Gewebe des Ösophagus sein, welches ebenfalls CDX-Transkriptionsfaktoren exprimiert.

Desweiteren präsentiert die vorliegende Arbeit Daten zur Regulation der Transportproteine organic solute transporter α und β (OSTα/β) durch die Transkriptionsfaktoren CDX1 und CDX2. Ich konnte zeigen, dass das Ausschalten der Expression von CDX1 und CDX2 zu einer Reduzierung von endogener OSTα/β–mRNA führt und eine Überexpression von CDX1 und CDX2 eine erhöhte Expression von OSTα-mRNA zur Folge hat.

Die Rolle, die CDX-Transkriptionsfaktoren in der intestinalen Differenzierung spielen sowie und ihre aberrante Expression in intestinalem metaplastischem Gewebe sind bereits gut erforscht. Jedoch war bisher wenig bekannt darüber, über welche exakten molekularen Mechanismen CDXs ihre aktivierende oder reprimierende Wirkung auf die Transkription von Genen ausüben. Das zweite Ziel meiner Forschungsarbeit war es daher, die mit CDX interagierenden Proteine, welche vermittelnd oder modulierend auf die Transkriptionsaktivität wirken, näher zu untersuchen.

Zu diesem Zweck habe ich eine Phagen-cDNA-Expressionsbibliothek mit Hilfe von poly(a)RNA, gewonnen aus humanen Dünndarm- und Dickdarm-Zellen, erstellt. Die Charakterisierung der Bibliothek bestätigte die hohe Qualität derselben. Die nichtamplifizierte Bibliothek enthielt $4.5\times10^6$ unabhängige Klone, wobei 99.6% der Phagenvektoren ein Insert trugen. Ich habe ein primäres Screening ($1.7\times10^6$ Klone per CDX-Protein) und ein sekundäres Far-Western-Protein-Interaktionsscreening mit *in vitro* translatiertem, $^{35}$S-gelabeltem, CDX1 und CDX2 durchgeführt. Das Screening führte zur Identifizierung mehrerer Klone, die potenziell für noch nicht bekannte Interaktionspartner für CDX-Proteine kodieren. Einer der vielversprechenden cDNA-Klone kodiert für das BRD1 Protein, welches ein Bromo-Domäne enthält, eine Proteinstruktur, die häufig in Molekülen mit Ko-Aktivatorfunktion zu finden ist. Ich evaluiere nun derzeit, inwieweit die positiven Klone des Far–Western-Screenings für wahre Interaktionspartner von CDX-Proteinen kodieren.

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ABC transporter | ATP-binding cassette transporter |
| ASBT | apical sodium-dependent bile acid transporter |
| BE | Barrett's esophagus |
| BSEP | bile salt export pump |
| C/EBP | CCAAT enhancer-binding protein |
| CA | cholic acid |
| CAD | coronary artery disease |
| CBDL | common bile duct ligation |
| CDCA | chenodeoxycholic acid |
| COX2 | cyclo-oxygenase 2 |
| CREB | cAMP response element-binding |
| DCA | deoxycholic acid |
| EAC | esophageal adenocarcinoma |
| EGCG | epigallocatechin-3-gallate |
| EGFR | epidermal growth factor receptor |
| FXR | farnesoid X receptor |
| GEJ | gastroesophageal junction |
| GCA | glycocholic acid |
| HGD | high grade dysplasia |
| HNF | hepatocyte nuclear factor |
| IBABP | ileal bile acid-binding protein |
| IBD | inflammatory bowel disease |
| IHC | immunohistochemical |
| IL | interleukin |
| IM | intestinal metaplasia |
| IR-1 | inverted repeat-1 |
| JNK | Jun N-terminal kinase |
| LCA | lithocholic acid |
| LGD | low grade dysplasia |
| LPS | lipopolysaccharide |

| | |
|---|---|
| LRH | liver receptor homologue |
| MAPK | mitogen-activated protein kinase |
| MRP | multidrug resistance protein |
| MβCD | methyl-β-cyclodextrin |
| NTCP | $Na^+$-taurocholate cotransporting polypeptide |
| OATP | organic anion transporting polypeptide |
| OST | organic solute transporter |
| PBC | primary biliary cirrhosis |
| PEPT1 | peptide transporter 1 |
| PFIC | progressive familial intrahepatic cholestasis |
| PKC | protein kinase C |
| PMA | phorbol-12-myristate-13-acetate |
| PXR | pregnane X receptor |
| RAR/RXR | retinoic acid receptor/retinoid X receptor |
| SCMT1 | sodium-coupled monocarboxylate transporter 1 |
| SHP | small heterodimer partner |
| SREBPs | sterol regulatory element-binding proteins |
| TGFα | transforming growth factor-α |
| TNFα | tumour necrosis factor-α |
| UDCA | ursodeoxycholic acid |
| VDR | vitamin D receptor |

# CONTENTS

# 1 INTRODUCTION

## 1.1 Bile acid (BA) synthesis

BAs are synthesized as a result of cholesterol metabolism in the liver, and approximately 50% of cholesterol is eliminated by its conversion into BAs. BAs function as physiological detergents to facilitate the intestinal absorption of dietary fats and fat-soluble vitamins. BAs are synthesized via two pathways. Ninety percent of the BAs are made via the classic pathway that takes place in the liver. Here, cholesterol is first converted to 7α-hydroxycholesterol via the cholesterol-7α-hydroxylase (CYP7A1) - the first and rate-limiting enzyme (Fig. 1.1). The alternative/acidic bile acid synthesis, ie. the remaining 10 %, is primarily catalysed by the sterol 27-hydroxylase (CYP27A1) [1]. The latter pathway occurs when the classic synthesis pathway is blocked by low CYP7A1 activity [2]. The synthesis of BAs from cholesterol consists of 15 enzymatic steps [3].

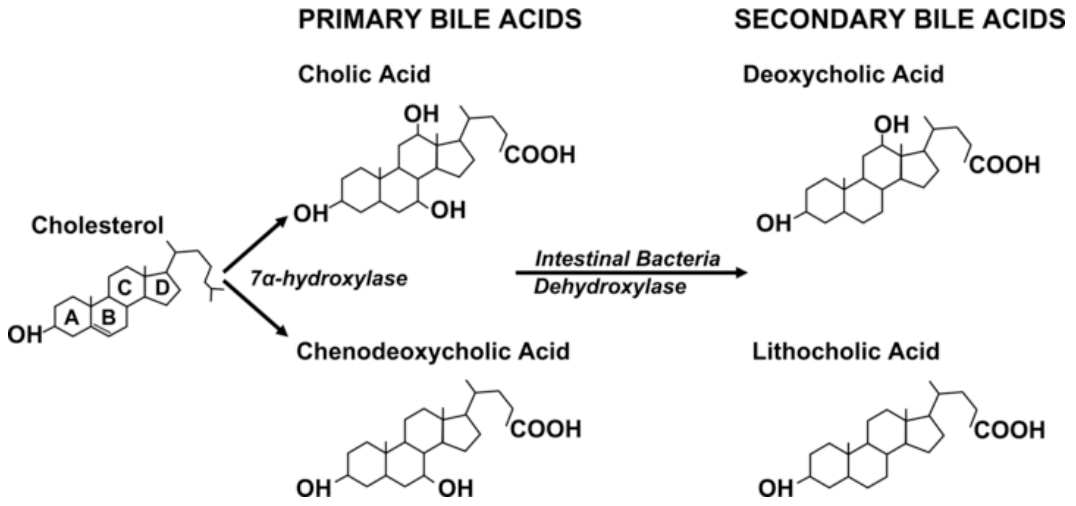



**Fig. 1.1 From cholesterol to BAs.** Conversion of cholesterol to BAs is initiated with the modification of sterol ring and is followed by side chain cleavage. Human primary BAs

formed from the cholesterol in the liver are cholic acid (CA) and chenodeoxycholic acid (CDCA). Secondary BAs are formed in the colon by bacteria, and include deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA). Taken from Khurana *et al* [4].

## 1.2 Enterohepatic circulation of BAs

Healthy human adults have approximately 3-4 g of BAs, which circulate 6-8 times/day between the liver and intestine. During the fasting stage, BAs are stored in the gallbladder, from which they are released into the intestine upon food intake, in order to aid digestion of lipids and fats into micelles. Approximately 95 % of BAs are reabsorbed actively by the apical sodium-dependent bile acid transporter (ASBT; *SLC10A2* gene) in the terminal ileum via active sodium-dependent uptake. Within the enterocytes, BAs bind to the cytoplasmic transporter protein ileal bile acid-binding protein (IBABP), and are exported into the portal venous blood via the heterodimeric membrane transporter organic solute transporter (OST) -α and OSTβ on the basolateral membrane [5, 6]. In the small intestine and colon, the primary BAs are deconjugated after exposure to the gut flora. In the liver, BAs are taken up mainly by the $Na^+$-dependent taurocholate-cotransporting polypeptide (NTCP; *SLC10A1*) on the sinusoidal hepatocyte membrane [7], whereas organic anion-transporting polypeptides (OATPs, *SLCO*s) are responsible for the minor sodium-independent bile acid uptake into hepatocytes. At the canalicular membranes of hepatocytes, BAs are exported to the bile via the ATP-dependent bile salt export pump (BSEP; *ABCB11*). Also present on the canalicular side, are other ATP-binding cassette (ABC) transporters, namely the multidrug resistance-associated protein-2 (MRP2; *ABCC2*). The flow of BAs from the small intestine via the liver to the gallbladder completes their enterohepatic circulation (Fig 1.2). This is an efficient and tightly regulated uptake and efflux process: less than 5 % of the intestinal BAs escape from reabsorption into the systemic circulation, and are released into the faeces.

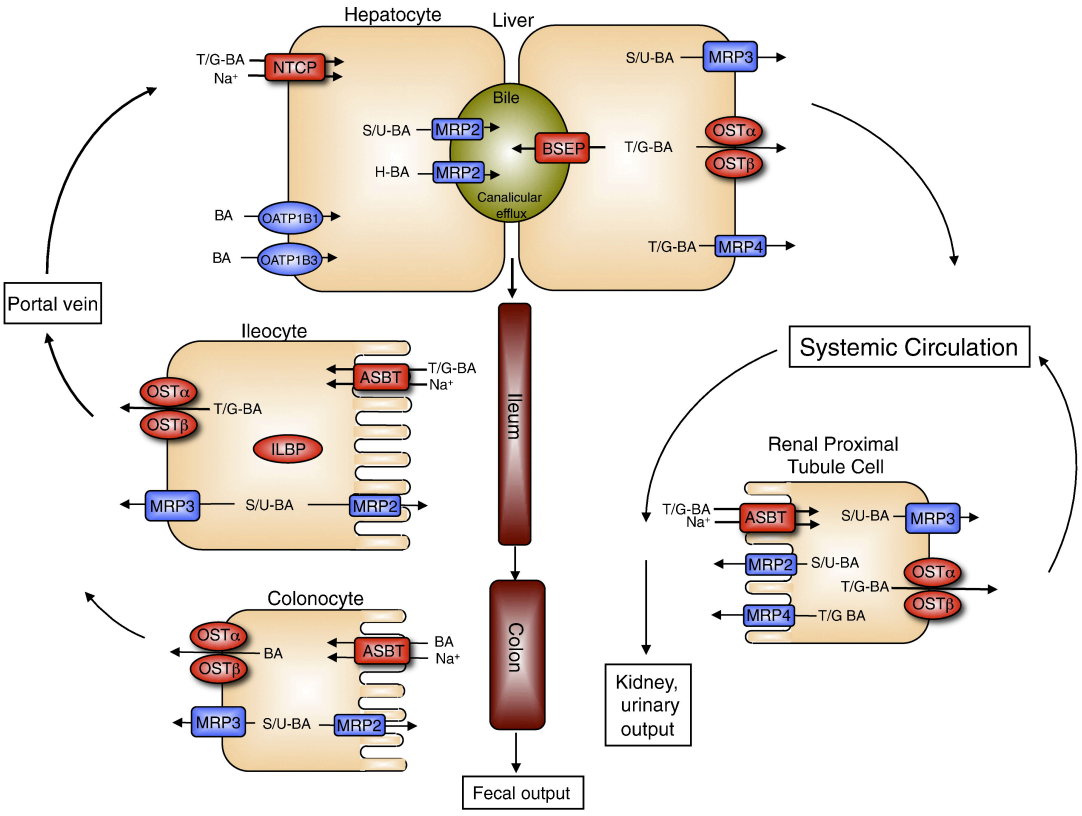



**Fig. 1.2 Overview of the enterohepatic circulation and systemic circulation.** BAs undergo efflux into the portal blood via the heterodimeric OSTα/β at the basolateral membrane of enterocytes. Together with the basolateral BA uptake system NTCP at the sinusoidal membrane of hepatocytes and BSEP at the liver canalicular membrane. ASBT and OSTα/β maintain efficient enterohepatic BA circulation and cholesterol homeostasis [8, 9]. BAs that are not absorbed in the ileum are reabsorbed at the colon. Spilled BAs from hepatocytes are reabsorbed by the renal proximal tubule cells, returned back to liver, and resecreted into bile. Taken from Dawson *et al* [10].

## 1.3 Regulation of BA transport

### *1.3.1 Nuclear receptors for BAs*

The expression levels of membrane transporters alter under different physiological and pathological conditions. In addition to their detergent function, BAs are signalling molecules. The farnesoid X receptor (FXR, *NR1H4*) was the first nuclear receptor for BAs identified, and is strongly activated by the BA ligand CDCA, as well as the secondary BAs LCA and DCA [8, 11, 12]. FXR is expressed in the liver, kidney, intestine, and adrenal gland. In most cases, FXR forms heterodimers with another nuclear receptor, the retinoid X receptor-α (RXRα; *NR2B1*) in order to bind to FXR response elements and to transactivate target genes [13]. The FXR:RXRα heterodimer can induce the expression of a repressor nuclear receptor, the small heterodimer partner (SHP; *NR0B2*), which consequently represses *CYP7A1* promoter activity, thereby inhibiting BA synthesis. This forms a negative feedback loop existing to prevents cells from becoming overloaded with BAs (reviewed in [14]).

BA transporter genes are under strict transcriptional regulation. FXR plays an important role in hepatic BA homeostasis. In wild-type mice, upon feeding with 1 % CA, Ntcp, and Cyp7a1 expression are downregulated, while Bsep expression is upregulated [15]. In Fxr knockout mice, no change in the Ntcp and Bsep expression is observed in the liver. Under cholestatic conditions, where there is significant accumulation of BA in the hepatocytes, FXR acts by inducing BSEP expression in order to increase bile efflux from hepatocytes, as well as inhibiting BA uptake via NTCP [16]. Expression of the BA efflux system OSTα/β is also upregulated via the bile acid receptor FXR in the human liver and intestine [17, 18].

Vitamin D receptor (VDR; *NR1I1*), also known as the calcitriol receptor, is activated by its natural ligand, 1,25-dihydroxyvitamin $D_3$ [1,25(OH)$D_3$], as well as by the secondary BA LCA [19, 20]. Similarly to FXR, upon ligand binding, VDR heterodimerizes with RXRα in order to bind downstream target gene promoters, such as the *CYP3A* promoter [20].

Pregnane X receptor (PXR; *NR1I2*) was first cloned from mice and described in 1998 [21], and is activated by naturally occurring steroids. The BAs LCA and 3-keto-LCA are also potent activators of PXR. Studies in Pxr-null mice have demonstrated that, Pxr has a protective role against liver damage, because activation of Pxr by LCA represses Cyp7a1 and thus reduces BA production [22].

## 1.4 BA signalling via other pathways

Increasing evidence suggests that BAs also function as signalling molecules in further physiological processes, in addition to their well-established roles in lipid absorption and cholesterol homeostasis. BAs interact with multiple signalling pathways beyond nuclear receptors, and can regulate triglyceride, cholesterol, energy, and glucose homeostasis [23]. BAs can activate the Jun N-terminal kinase (JNK)/c-Jun cascade in rat primary hepatocytes and consequently downregulate Cyp7a1 and BA synthesis [24]. DCA has also been shown to activate the extracellular signal-regulated kinase (ERK) pathway, as well as CCAAT-enhancer-binding proteins (C/EBPs), the cAMP response element-binding protein (CREB), and c-Jun signalling, as hepatocyte-protective mechanisms [25].

In addition to BA-specific nuclear receptors described above, BAs have also been shown to interact directly with the membrane-type rhodopsin-like G-protein-coupled BA receptor (GPCR) TGR5 (also known as M-BAR) [26]. TGR5 is expressed in brown adipose tissue, spleen, macrophages, lung, fetal liver, colon, and ileum, and it plays a role in macrophage function in BA-induced immune responses [27]. The absence of Tgr5 in an animal model has revealed its role in intestinal homeostasis via regulation of intestinal permeability [28].

## 1.5 ASBT

### *1.5.1 General aspects*

The gene encoding the ASBT (gene symbol *SLC10A2*) is located on the human chromosomal position *13q33* [29]. It was first cloned from a hamster intestinal cDNA library [30]. This was followed by cloning of the human [31], rat [32], rabbit [33], and mouse [34] ASBT/Asbt homologues. The *ASBT* gene encodes a 48 kDa transmembrane protein expressed in the terminal ileum, biliary tract, and renal proximal tubule cells. Structural information of human ASBT membrane topology is not clear: membrane insertion scanning has predicted a nine transmembrane model [35], whereas using dual label epitope insertion scanning mutagenesis has a predicted seven transmembrane topology [36]. Crystal structure of the bacterial *Neisseria meningitidis* homologue of ASBT consists of 10 transmembranes (Fig. 1.3) [37]. BAs are transported into the cell at a stoichiometry of two sodium ions per one BA molecule [38]. At the apical membranes of ileal enterocytes, ASBT is the chief mediator of active sodium-dependent intestinal BA absorption [39] and it has a higher affinity for unconjugated BAs (CA, DCA, CDCA, and UDCA) than conjugated BAs or taurocholate [40].

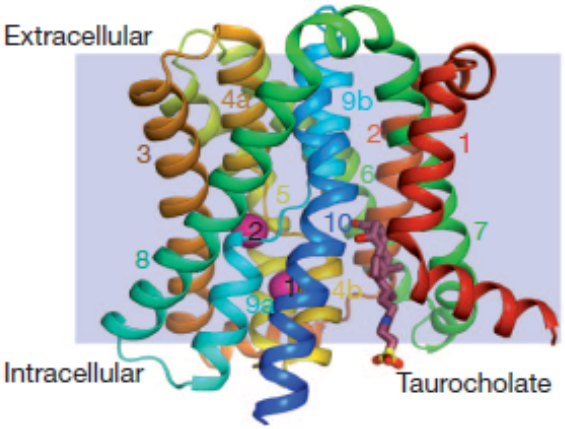



**Fig. 1.3 Crystal structure of the bacterial (*Neisseria meningitidis*) homologue of ASBT.** A ribbon representation of an ASBT homologue consists of 10 transmembranes, with red colour at the N-terminus and blue colour at the C-terminus. Pink spheres depict the sodium position 1 and 2, and the position of taurocholate is indicated in dark pink. Taken from Hu *et al* [37].

### *1.5.2 Regulation of ASBT by BA*

In Asbt$^{-/-}$ mice [41], the BA circulation is disrupted, and Cyp7a1 expression is increased 2.7-fold in male mice and 5.2-fold in female mice. The regulation of ASBT expression by BAs has remained somewhat controversial, given that the experiments have been conducted in different species and cell lines. Chen *et al* have shown that mice fed with BAs exhibit decreased Asbt expression at the protein and mRNA level, and have proposed that this negative feedback mechanism is mediated by Fxr via short heterodimer partner (Shp)-dependent repression of the *Asbt* promoter [42]. In rabbits, DCA feeding represses Asbt expression via elevating Shp levels in the ileum [43].

### *1.5.3 Non-BA-dependent transcriptional and post-transcriptional regulatory mechanisms of ASBT expression and function*

Prior studies have shown that *ASBT* gene expression is controlled at the transcriptional level in a complex manner: Known transcriptional regulators of the human *ASBT* promoter include the glucocorticoid receptor (GR) [44] and hepatocyte nuclear factor-1 α (HNF)-1α [45]. Consistent with the latter, Hnf-1α knockout mice show reduced Asbt mRNA and protein levels, implying that Hnf-1α is involved in maintaining the basal expression level of Asbt [46]. Other regulators of the *ASBT*/*Asbt* promoter include the peroxisome proliferator-activated receptor-α (PPARα) [45] and, in rats, VDR [47]. Both serum [48] and certain BAs [49] appear to elevate ASBT expression via activator protein-1 (AP)-1. In addition, cholesterol has been shown to modulate human ASBT expression in Caco-2 cells: the cholesterol derivative 25-hydroxycholesterol inhibits the ASBT expression at the mRNA and promoter level [50] via the transcription factors sterol regulatory element-binding protein (SREBP)-2 and HNF-1α [51]. However, this effect has been contradicted in further cholesterol-feeding animal models: no change of ASBT expression is observed in rats, whereas its expression is induced in rabbits [52]. Treatment with inflammatory cytokines interleukin (IL)-1β and tumour necrosis factor (TNF)-α significantly reduces human *ASBT* promoter activity, and this effect is mediated by the transcription factor c-Fos [53]. At the post-transcriptional level,

ASBT expression is reduced by cholesterol depletion with methyl-β-cyclodextrin (MβCD) treatment, significantly reducing its interaction with lipid rafts [54]. Treatment with phorbol-12-myristate-13-acetate (PMA) decreases the ASBT transport activity via the protein kinase C (PKC) pathway [55]. In a knockout mouse model, the transcription factor Gata4 regulates the jejunal-ileal gradient of Asbt expression, which provides a molecular mechanism for the tissue-specific expression of Asbt in the terminal ileum [56].

### *1.5.4 Pathophysiology*

Alterations in ASBT function or *ASBT* gene expression have been associated with intestinal diseases in humans: Mutations in the *ASBT* gene can lead to BA malabsorption [57], whereas reduced ileal ASBT protein [44] or mRNA [58] levels have been found in patients with inflammatory bowel disease (IBD).

### *1.5.5 Genome-wide studies and genetic polymorphisms of ASBT*

Genome-wide association and single nucleotide polymorphism (SNPs) studies have been reported for ASBT [59]. A genetic variant allele of the *ASBT*, *rs9514089*, is presented as a risk factor for male non-obese gallstone carriers [60], although in a more recent study in 2011, the same allele showed no association in a smaller cohort [61]. Therefore future larger cohort studies are needed to confirm these findings. A systematic study of the association between coding SNPs and the taurocholate transport function of ASBT, has shown a significant impairment for 292*G>A* and 431*G>A*, whereas a rare novel variant 790*A>G* leads to the near complete loss of transport activity in comparison with the wild type [62].

### *1.5.6 ASBT inhibitors as therapeutic options*

Therapeutic drugs have been developed to inhibit ASBT activity in an attempt to lower serum cholesterol level [63]. The ASBT inhibitor (264W94) has been proposed as a candidate for the therapy of type 2 diabetes mellitus [64]. In a randomized clinical trial, another ASBT inhibitor, A3309, has been shown to be effective for treatment of chronic idiopathic constipation [65]. A novel monoclonal

antibody has been synthesized to target ASBT specifically, leading to the conversion of more cholesterol into BAs, thus making it a promising agent for treatment of hypercholesterolemia [66]. Interestingly, (-)-epigallocatechin-3-gallate (EGCG), a component of green tea, has been shown to inhibit ASBT, by altering the association of ASBT with lipid rafts on the plasma membrane [67]. Determination of the crystal structure of the *Neisseria meningitides* bacterial homologue of human ASBT may further advance the design of more potent ASBT inhibitors [37]. However, potential drug-induced side effects of ASBT inhibitors have been reported: For example, increased free BA concentrations following ASBT inhibitor treatment may be a major cause for diarrhea, gallstone disease, and hypertrigyceridemia [68].

Lowering the blood cholesterol content, especially low density lipoprotein (LDL) which is known to be atherogenic, has been a target in reducing the risk for vascular atherosclerosis. Several studies have revealed the potential contribution of BAs to cardiovascular conditions. For example, when comparing the BA excretion levels in patients with coronary artery disease (CAD) and CAD-free patients, the former group had a lower amount of total BA. Therefore, the BA excretion level may act as a predictor for CAD [69]. Blocking ASBT-mediated bile acid uptake with specific inhibitors can reduce serum cholesterol level and prevent atherosclerosis in animal models, for example in rabbits [70], apoE (-/-) mice [71], and in guinea pigs [72].

## 1.6 OSTα/β

### *1.6.1 General aspects*

Whereas ASBT on the apical membrane of ileocytes is responsible for BA uptake, the protein(s) that transport BA across the basolateral membrane of ileocytes to the portal blood remained unknown until 2001, when Wang *et al* first cloned OSTα/β from the sea animal little skate (*Leucoraja erinacea)*, by expression profiling of its liver cDNA library [73]. The human and mouse orthologues were subsequently cloned by the same group in 2003 [74]. The *OSTα* and *OSTβ* genes are located on different chromosomes, at positions *3q29* and *15q22*, respectively. Their transport

activity of the heterodimer is sodium-independent, saturable, and unaffected by intracellular ATP depletion [5]. In the case of estrone 3-sulfate transport, human and mouse OSTs/Osts cannot mediate transport when expressed individually in *Xenopus* ooctyes, transport only occurs when both subunits are coexpressed on the membrane [74]. The OSTα/β heterodimer is responsible for BA efflux into the portal blood in the ileum and other tissues where ASBT is expressed; it also transports estrone 3-sulfate, dehydroepiandrosterone 3-sulfate (DHEAS), digoxin, prostaglandin E2 [5], and steroids [75]. OSTs are predominantly found at the mRNA level in the human testes, colon, liver, small intestine, kidneys, ovaries, and adrenal glands. Human OSTs are highly expressed in liver, whereas mouse Osts are expressed at low levels in the liver [76]. In mice, Ostα has a protein size of 50 kDa, although minor protein species bands can also be observed at around 80 kDa - the predicted molecular weight is 37.8 kDa. The mouse Ostβ forms two protein species at 19 and 17 kDa, with a predicted molecular weight is at 14.7 kDa. The protein sizes of the human OSTα and OSTβ are 37 and 19 kDa respectively [77]. OSTα and OSTβ interact directly to form a heterodimer, which stabilizes the protein complex. When they are expressed separately, OSTs undergo rapid degradation. The main function of OSTβ is to act as a chaperone to move the OSTα protein from the endoplasmic reticulum to the plasma membrane [78]. A study in the Ost knockout mice, has provided *in vivo* evidence for the Ost function in BA circulation, and has emphasized the important role of OSTs/Osts in BA homeostasis [79]. For example, in these mice, the accumulated BAs in the intestine has been shown to upregulate FXR, which then increases the hormone Fgf15, leading to a decrease in hepatic BA synthesis.

### *1.6.2 Transcriptional regulatory mechanisms of OSTα/β*

*OSTα/β* are direct target genes of FXR. Human *OSTα/OSTβ* promoter-reporter constructs can be induced by BAs via the presence of FXR response elements, and consistent with this, treatment of ileal biopsies with CDCA leads to elevated OST mRNA levels [18]. In accordance, Ost*α/β* mRNA levels are induced in the intestine and liver upon Fxr agonist GW4064 treatment in wild type but not in the Fxr (-/-)

mice [80]. In mice, the *Ostα/β* genes are regulated positively by BA via Fxr response elements and negatively via response elements for the nuclear receptor liver receptor homologue-1 (Lrh1), a Shp target, located on the promoter regions of the two genes. This dual regulation by Fxr and Lrh-1 allows the *Ost* gene expression to be adjusted by BA via both positive and negative feedback [81]. Furthermore, the mouse *Ostα/β* promoters are regulated by the liver X receptor-α (Lxrα)/Rxrα heterodimer via inverted repeat-1 (IR)-1 elements in the promoter region, and in the case of the *Ostα* promoter, another nuclear receptor hepatocyte nuclear factor-4α (Hnf-4α) can further increase the effect [82]. It also appears that the *OST*/*Ost* promoters are regulated in an organ- and species-specific manner, because VDR/Vdr ligands reduce Ostα/β expression in rat jejunum, ileum, colon, and liver, but have no effect on OST expression in the human ileum and liver [83].

### *1.6.3 Pathophysiology*

In human cholestatic liver injury, the adjustment of BA transporter expression aims to protect the liver from excess and toxic BAs by restricting their uptake and inducing their efflux, in addition to the reducing bile acid synthesis [84]. The expression of OSTs is upregulated in human primary biliary cirrhosis (PBC), and similarly to the rodent counterparts upon common bile duct ligation (CBDL), this effect is FXR-dependent [17]. OST expression levels have been observed in non-obese female gallstone carriers in comparison with healthy control subjects [85]. Furthermore, the expression levels of OSTs strongly correlate with those for the BA receptor FXR in the entire study group.

## 1.7 Caudal-type homeobox-1 (CDX1) and -2 (CDX2)

### *1.7.1 General aspects*

CDX1, CDX2, CDX3, and CDX4 are transcription factors of the homeodomain family, and homologues of the *Drosophila melanogaster* protein Caudal that is essential for the early anterior-posterior development and body patterning [86]. Homeodomains are structurally conserved protein domains of ~60 amino acids and

contain helix-turn-helix motifs that can mediate protein binding to DNA in a sequence-specific manner. Many homeodomain transcription factors regulate patterns of anterior-posterior development in animals, fungi, and plants [87].

CDX1 and CDX2 were first identified and cloned by the Mallo group from genes differentially expressed between the colon carcinoma and adjacent normal mucosa. Human CDX1 shares 85 % homology with mouse Cdx1, and human CDX2 shares 94 % homology with the mouse Cdx2 [88]. Despite the high level of amino acid identity (95 %) within their DNA-binding domains, the two CDX factors play at least partially distinct roles in the intestine: $Cdx1^{-/-}$ mice exhibit a shift in the body frame [89], and $Cdx2^{+/-}$ heterozygous mutant mice develop colonic tumours [90]. These two mouse models also show differential responses upon inflammatory challenge [91]. Complete Cdx2-null mice are embryonically lethal, as evidently are Cdx1/Cdx2 double-null mice. In adult mammals, CDXs/Cdxs are expressed exclusively in the small and large intestine, playing important roles in the proliferation and differentiation of intestinal epithelial cells. However, their levels have been reported to be aberrantly elevated in esophageal metaplasia, such as BE, compared to the adjacent normal tissue [92, 93]. Similarly to the BA transporters, CDX2 expression has been reported to be decreased upon progression of esophageal metaplasia into high-grade adenocarcinoma [94, 95].

### *1.7.2 Transcriptional target genes of CDX1 and CDX2*

A number of CDX1 and CDX2 transcriptional target genes have been identified so far. These include marker genes for intestinal differentiation, such as *sucrase isomaltase* (*SI*) [96], goblet-cell-specific *mucin 2* [97], cell adhesion differentiation marker *cytokeratin 20* [98], *desmocollin 2* [99], *E-cadherin* [100], *lactase* [101], and *VDR* [102]. Certain CDX target genes encode intestinal transporters, such as the peptide transporter 1 (PEPT1; *SLC15A1*) [103] and the sodium-coupled monocarboxylate transporter 1 (SCMT1; *SLC5A8*) [104]. The observation that CDX2 regulates the *ABCB1* gene encoding the multidrug resistance protein 1

(MDR1) may have implications in gastrointestinal cancers [105]. Furthermore, it has been suggested that CDX1 and CDX2 can regulate their own expression in esophageal cell lines, via both auto- and crossregulation [106]. CDXs also regulate several intestine-specific genes, such as those encoding *SI* [96] and *NADPH oxidase* (*NOX1*) [107], via an evolutionarily conserved mechanism involving cooperation with HNF-1α and members of the GATA transcription factor family. Furthermore, CDX2 can synergize with HNF-1α, leading to activation of the gene encoding the brush-border enzyme lactase-phlorizin hydrolase (LPH) [108].

### *1.7.3 Role of CDXs in gastric metaplasia/carcinogenesis*

Gastric cancer develops through the following stages: normal gastric mucosa>gastritis>gastric atrophy>gastric cancer. Normal stomach does not express either CDX1 or CDX2. CDX1 and CDX2 have been consistently detected in gastric intestinal metaplasia (IM) by immunohistochemical (IHC) staining and RNA analysis [109-111]. CDX2 is also closely associated with gastric carcinoma differentiation status [112]. Expression of the homeobox gene CDX2 precedes that of CDX1 during the progression of IM [112]. The above evidence suggests that CDX1 and CDX2 are involved in the development of gastric IM, which is also highlighted in transgenic Cdx1 and Cdx2 mice. In Cdx2 transgenic mice, normal gastric mucosal cells are replaced by IM on day 37, and gastric mucosal cells are transformed into enterocytes, goblet cells, and enteroendocrine cells, indicating that Cdx2 is an important transcription factor in this conversion process [113]. Furthermore, endogenous Cdx1 expression is induced by Cdx2 in IM of the stomach [114]. In Cdx1 transgenic mice, normal gastric mucosa was replaced by all four intestinal epithelial lineages; in this case, also Paneth cells are found. Pseudopyloric gland metaplasia, induced in Cdx2 transgenic mice, is not observed in Cdx1 transgenic mice. Cdx1 mice also have thicker metaplastic mucosa than Cdx2 mice. Overall, these data suggest that Cdx1 and Cdx2 can independently transform the normal gastric epithelium into epithelium of intestinal characteristics, although to a different extent in terms of degree of differentiation, structure, and proliferation [115]. CDX2

serves as a sensitive marker for intestinal-type gastric carcinoma and CDX2-positive patients show a better prognosis than CDX2-negative patients [116].

### *1.7.4 The role of CDXs in colon cancer*

The tumour suppressor role of Cdx2 was first suggested in a mouse model in which $Cdx2^{-/-}$ mice died at birth, and the $Cdx2^{+/-}$ mice developed intestinal polyps [90]. The reduction or complete loss of CDX1 and CDX2 has been found in human colorectal carcinoma tissues compared to adjacent normal tissues and colon cancer-derived cell lines [88], suggesting that CDXs participate in the pathogensis of colon cancer. In $Cdx2^{+/-}$ mice, reduced Cdx2 expression does not initate colon tumor, but facilitates the tumour progression upon DNA mutant challenge [117]. By overexpressing CDX2 in a colon cancer cell line HT29, Mallo *et al.* have found that CDX overexpression reduces the cell growth and tumorigenicity, and inhibits cell migraton [118]. In colon cancer, there are other signalling pathways involved in regulating CDXs. In colon cancer cell lines with constitutively activated Ras, CDX2 is downregulated via PKC, which correlates with PKC-dependent depletion of c-Jun, whereas Ras induces the CDX1 expression via a MEK1/MAPK- dependent pathway [119]. *Phosphatase and tensin homologue* (*PTEN*) is one of the most commonly mutated tumor suppressor genes in human, and acts through its phosphatase protein product. Mutation or depletion of PTEN results in an increase of cell proliferation and inhibition of cell apoptosis. Kim *et al* have shown a similar distribution of Cdx2 and Pten in wild-type and $Pten^{+/-}$ mice. They subsequently showed that Pten can stimulate Cdx2 at the mRNA and protein level via the PTEN/P13K pathway, and that proinflammatory cytokine TNF-α reduces CDX2 expression via altering the balance of NF-κB subunits p50 and p65 [120]. In colorectal cancer cells, oncogenic activation of β-catenin can stimulate CDX1 expression [121]. Overall, restoring the CDX1 and CDX2 expression may prove to serve as an effecive therapy against colon cancer.

## 1.8 BE

### *1.8.1 Definition*

BE was first described by Dr. Norman Barrett in 1950. The definition of BE has been a topic of debate (Fig 1.4). According to the American Gastroenterology Association (AGA) Institute Medical Position in 2011, BE is suspected when "under endoscopic examination, columnar epithelium is observed to extend above the gastroesophageal junction (GEJ) into the tubular esophagus, BE can be defined conceptually as the condition in which any extent of metaplastic columnar epithelium that predisposes to cancer development replaces the stratified squamous epithelium that normally lines the distal esophagus" [122].

The presence of IM containing goblet cells is critical for the diagnosis of BE. Disagreement between professional societies raises the question of whether it is important to include goblet cells in the diagnostic criteria. In the Japanese and British guidelines, goblet cells are not essential to establish a diagnosis of BE, largely because of sampling error, and difficulties in distinguishing true goblet cells and pseudo-goblet cells in pathological terms. It is worth noting that IM without goblet cells also predisposes a patient to develop esophageal adenocarcinoma (EAC), therefore, it is important to find a non-morphological marker.

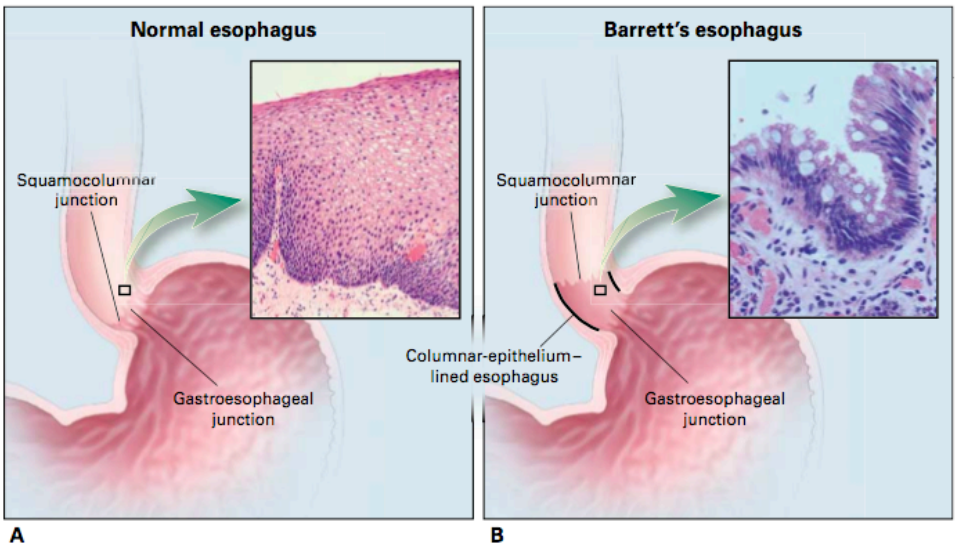


**Fig. 1.4 BE.** A. Normal esophagus: squamous epithelial lining of the normal esophagus, B. Magnified view of BE tissue shows the columnar lining containing goblet cells. Taken from Spechler and Goyal [123].

### *1.8.2 Epidemiology*

In Western countries, BE is present in 1.6 % of the general adult Swedish population [124]. A cohort study using the general practice research database from the UK reported a rise of incidence from 19.8/100,000 (~0.02 %) in 1997 to 40.5/100,000 (~0.04%) in 2002. The variation in the reported incidences between countries may due to the sample population selection and the definition used for BE. However, the authors speculate that this rise could be explained by increased awareness of BE and improved endoscopic diagnostic skills of endoscopists [125]. Although the prevalence of gastroesophageal reflux disease is increasing in Asia, the prevalences of BE and EAC has remained low in most Asian compared to Western countries. The prevalence varies between countries (reviewed in [126]): In China, a prevalence of 1 % was found in patients who complained of upper gastrointestinal symptoms, and in this study population, age, and esophagitis were further associated with BE [127], Japan has been reported to have the prevalence of BE of 19.9 % in patients who undergo esophagogastroduodenoscopy [128]. In Korea, BE prevalence was reported to be 0.22 % in a retrospective study [129]. However, in a prospective multicenter-based study in Korea, BE was shown to present in 0.84 % of study population [130].

Overall, the increase of BE prevalence in Asia has probably been due to high-fat diet and changes in lifestyle. The west and the east do share common risk factors such as male sex, older age, and long duration of reflux symptoms.

### *1.8.3 Risk factors*

#### 1.8.3.1 Gastroesophageal reflux disease (GERD)

GERD is the primary predisposing risk factor for BE that affects approximately 20 % of the population in developed countries. About 10-15 % of patients with GERD develop BE [123], which predisposes to EAC: patients with BE are 30-40 times more likely to develop EAC [131].

#### 1.8.3.2 Alcohol

Alcohol is a well-established carcinogen responsible for the pathogenesis of many cancers. A recent population-based case-control study reports that life-long alcohol consumption does not increase the risk of BE, and increased beer intake might in fact have a protective role against BE [132]. Similarly, other previous studies also have shown that alcohol is not a risk factor for BE, and in this case, recent alcohol consumption was also taken into account in the analysis [133]. A prospective cohort study in the Netherlands has shown no correlation between alcohol consumption and BE [134]. A community-based study has found that the risk of BE was not associated with consumption of liquor or beer, and inversely associated with wine intake [135].

#### 1.8.3.3 Smoking

A US-based prospective study has shown an increased risk of BE in former smokers, and is of the highest risk in heavy and remote smoking [136]. In another study, former smokers, but not current smokers were found to be at higher risk of developing BE [134]. Smith and colleagues also have shown that smokers have a higher risk of developing BE compared to non-smokers [137]. In contrast, Kubo and colleagues have not identified smoking as risk factor for BE [138].

## 1.9 Current theories of molecular origin of BE

The first theory suggests a role for stem cells in the pathogenesis of BE. The intestinal stem cell marker leucine-rich repeat-containing G protein-coupled receptor

5 (LGR5) has been been found to be overexpressed in BE, although the intensity of staining is higher in EAC than BE [139].

The second theory suggest that bile salts and acids in the reflux contents of GERD cause the tight juctions to break in squamous cells, and allow leakage of the refulx contents into the basal layer. These factors can induce CDX1 and CDX2 transcription factors, causing esophageal cells to transdifferentiate into columnar cells (reviewed in [140]), as shown in Fig 1.4.

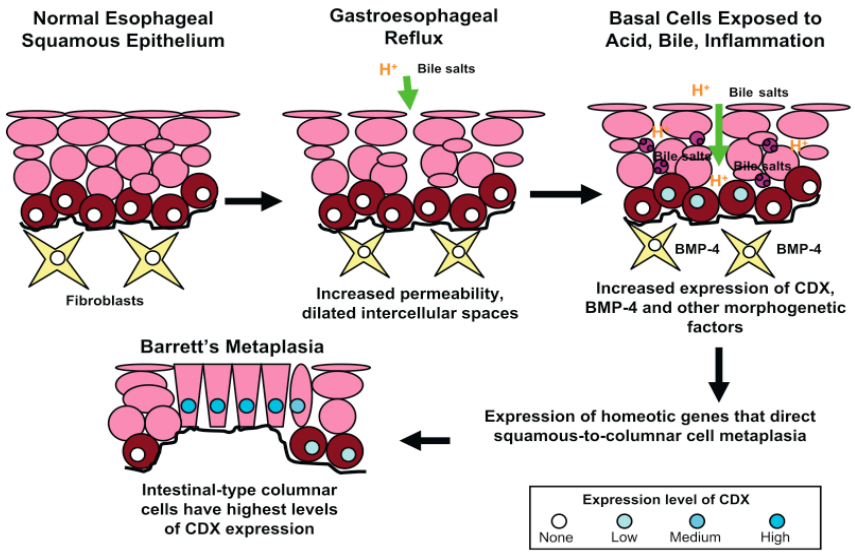



**Fig. 1.5 Transformation of squamous esophageal epithelium to BE epithelium.** This picture shows the transformation of normal squamous esophageal epithelium into BE. After chronic exposure to bile salts and acids, the tight junctions become loose, which allows bile salts in and the transformation of normal esophageal cells into intestinal-like cells. Taken from Scouza *et al* [140].

The third theory concerns p63, a homologue of the tumor suppressor protein p53. It is expressed in the basal or progenitor layers of many epithelial tissues. P63 can act as a potent inducer of apoptosis. Stem cells in the basal layer of stratified squamous epithelia express high levels of p63 and undergo asymmetric division to enable both self-renewal and progression to a stratified multilayer lineage. The latter cells may express less p63, and are capable of limited proliferation and self-renewal, but are

ultimately destined for terminal differentiation. The absence of p63 results in failure to maintain a basal cell population, suggesting a requirement for p63 in the regenerative aspect of stem-cell division and for maintenance of a normal stratified squamous esophageal epithelium [141]. Wang *et al*. have studied the molecular origins of the role of embryonic cells as precursors of Barrett's-like metaplasia. The study set out to look in a p63-null mouse model, where stem cells undergo self-renewal in the absence of any mutations. They found that upon programmed damage, embryonic cells compete with cells in the proximal stomach, and migrate and replace the damaged squamous cells [142]. This contradicts a previously proposed mechanism, where metaplasia is formed prior to the damage by transformation of the existing tissue [140]. The results of this study have important implications for understanding the underlying cause of other gastrointestinal malignancies, such as gastric and pancreatic cancer, and IBD [142].

## 1.10 BAs and BE

Excess levels of BAs are toxic and have been shown to be carcinogenic to the esophagus, stomach, colon, and liver [143]. In the colon, high levels of DCA can induce colon carcinoma in mice [144]. BAs are a major component of duodenal juice, which can cause severe esophageal mucosal damage [145]. BA can cause oxidative damage, apoptosis, and DNA damage. Acidic bile salts can modulate the squamous epithelial barrier by altering the expression levels of claudin 1 and claudin 4 [146]. Nehra *et al* first suggested the damaging effects of BAs in the esophageal mucosa. The predominant BAs are CA, TCA and GCA, but in patients with erosive esophagitis, BE and esophageal stricture, there is a significantly greater proportion of secondary BAs, DCA and TDCA [147]. Furthermore, bile salts and acids exhibit different effects on cell proliferation rate. Bile salts alone increase proliferation, whereas addition of BAs inhibits proliferation [148].

Xenobiotic-sensing nuclear receptor PXR was found overexpressed in EAC and BE in comparison to normal squamous epithelium [149]. Moreover, cytoplasmic staining

of PXR was observed in BE, whereas nuclear staining was seen in EAC. The translocation of PXR from cytoplasm to nuclei was confirmed in an esophageal cell line OE19, induced by LCA or rifampicin [150]. FXR is also overexpressed in BE compared to normal esophageal mucosa and decreased in EAC, and the FXR antagonist guggulsterone induces apoptosis in BE cell lines [151]. A similar study has shown that, in addition to FXR, the levels of its target genes *SHP* and *IBABP* are increased in BE columnar epithelium compared to normal squamous epithelium, and FXR and IBABP levels can be induced exogenously by DCA in esophageal cell lines. In the same study, interleukin-8 (IL-8) and macrophage inflammatory protein-3α (MIP3α) levels are also elevated. These data suggest that BAs can induce an immune response [152]. To further emphasize the role of BA transporters in BE pathogenesis, Dvorak *et al.* have demonstrated that ASBT, IBABP, and MRP3 are elevated in BE and decreased in EAC, at both the mRNA and protein levels [153]. This is another adaptive response mechanism of the columnar mucosa against GERD. The BA receptor TGR5 was found to be expressed in BE and EAC, whereas only a minimal amount can be detected in normal esophageal mucosa. TGR5 mediates TDCA-induced expression of the NADPH oxidase isoform NOX5-S and $H_2O_2$ production [154]. Overall, these findings illustrate the local cellular changes that take place in response to the contents of chronic reflux.

## 1.11 Transcription factors associated with BE: p53, NF-κB, CDX1, and CDX2

Overexpression of the p53 protein has been reported in patients with BE [155]. Weston *et al.* have reported that p53 protein overexpression is more significantly associated with BE progression to high-grade dysplasia [156]. In a similar study by Skacel *et al.*, p53 positivity was correlated with progression to high-grade dysplasia/carcinoma [157].

NF-κB is a transcription factor that controls cell proliferation and survival. NF-κB is activated in EAC and its absence can be used to determine response to neoadjuvant

chemotherapy and radiation therapy, and low pH and DCA can induce NF-κB DNA-binding activity [158]. In an OE33 cell line-based study, DCA can cause DNA damage, and can also induce NF-κB activity [159].

CDX2 is expressed in patients with BE and inflammatory esophageal mucosa [125, 126]. Several studies have confirmed the elevated expression of CDX1 and CDX2 in BE and inflammatory esophageal mucosa in comparison to normal esophageal mucosa, at both the mRNA and protein levels [160]. In a microarray study, CDX1 and CDX2 expression were found to be differentially upregulated in BE, a finding also confirmed using IHC [161]. These data suggest that CDXs are involved in the pathogenesis of BE. Furthermore, CDX1 and CDX2 expression were induced in a bile acid-specific manner. DCA and CA show strongest activation of the CDX2 promoter via NF-κB, whereas the remaining BAs have no stimulatory effect [162]. As demonstrated in a rat model of BE, mixture of BAs, including CA, GCA, and TCA, can activate CDX1, and in esophageal derived cell lines, CDX1 and CDX2 exhibit homologous auto-regulation and heterologous inter-regulation [106].

## 1.12 Other cellular changes in BE

Other cellular changes in BE include alterations in the cell cycle, cellular architecture, and proliferation rate. Cyclin D1 expression is increased at the protein level [163], and this may prove to be a useful predictive biomarker for the development of EAC in BE patients [164]. The antigen Ki-67 is a nuclear protein marker of cell proliferation, and can be detected in the cell nuclei during interphase. Nuclear localization of Ki-67 in BE with low grade dysplasia (LGD) can be found in the lower crypt, whereas in BE with high grade dysplasia (HGD) the Ki-67 protein is found located in the upper crypt and surface epithelial zones [165]

## 1.13 Helicobacter pylori and BE

*H. pylori* is a Gram-negative bacterium and is a major cause of duodenal and gastric ulcers and stomach cancer. Vaezi *et al.* showed that colonization with CagA-positive strains of *H. pylori* is more common in healthy people and GERD patients than in BE patients, which suggests a protective role against malignant disease [166].

## 1.14 Cyclo-oxygenase 2 (COX2) and BE

COX2, or prostaglandin endoperoxide synthase 2, is encoded by *PTGS2* gene, and is involved in prostglandin synthesis. COX2 is constitutively expressed in most cells under normal conditions, and its expression can be induced by inflammation. Elevated COX2 and inducible nitric oxide synthase (iNOS) mRNA and protein levels in BE indicate their involvement in early pathogenesis [167], and the levels increase during progression from low-grade to high-grade dysplasia [168]. In addition, pulses of acids or BAs enhances COX2 expression in BE tissue explants, which can be attenuated by the COX2 inhibitor NS-398 [169]. Similar findings have been shown in Barrett's-associated adenocarcinoma cell lines [170]. Another COX2 inhibitor, rofecoxib, is active against esophageal dysplasia and adenocarcinoma [171]. COX2 expression is negatively associated with the survial of patients undergoing surgery for EAC [172].

## 1.15 Inflammation in BE

Inflammatory pathways are associated with the development of BE. Proinflammatory cytokines IL-8 and IL-1β are elevated in esophagitis, BE, and EAC [173]. DCA activates the transcripton factor NF-κB at neutral pH and subsequently its downstream targets IκB and IL-8 in a BE cell line OE33 [174].

TNF-α is a cytokine released by immune cells and other cell types, causing inflammation. There is an increase in TNF-α expression in the progression of Barrett's metaplasia to adenocarcinoma, and upregulation of TNF-α induces

expression of the c-myc oncogene via β-catenin in an NF-κB-independent manner [175].

## 1.16 Signalling pathways in the pathogenesis of BE

It is well established that multiple cell signalling pathways are altered during the course and progression of BE. Elucidating the altered pathways during disease progression may provide insight into future treatment or adjuvant treatment strategies.

Epidermal growth factor receptor (EGFR) exists on the cell surface and is activated by binding of its specific ligands, including EGF and transforming growth factor-α (TGF-α). Both EGFR and TGF-α are expressed in the inflamed tissue in BE [176, 177]. DCA can induce CDX2 expression in esophageal cells via the EGFR signalling pathway [178]. EGFR expression has been shown to increase as the disease progresses form BE, to low-grade dysplasia, high-grade dysplasia, and EAC, which makes it a potential biomarker candidate for predicting esophageal cancer risk [179].

The MAPK signalling pathway plays roles in cellular differentiation, development, transformation and apoptosis. Patients with GERD have a lower level of ERK1/2 phosphorylation, and acid exposure can increase ERK1/2 expression levels and their phosphorylation status [180]. Kinome profiling of BE and paired squamous esophageal tissue has shown that EGFR activity was decreased in BE compared to normal squamous tissues, and MAPK is downregulated in BE in comparison to its surrounding tissues [181].

BRAF and KRAS2 mutations are found in Barrett's adenocarcinoma, implying that disruption of the RAF/MEK/ERK (MAPK) kinase pathway is involved in the early development of BE [182]. The transforming growth factor-β (TGF-β) is constitutively expressed in healthy esophageal epithelium, but becomes progressively more overexpressed in inflamed GERD tissue, metaplastic BE epithelium, and EAC

[183]. The Ski and SnoN proteins negatively regulate TGF-β via interaction with the SMAD complex and recruitment of histone deacetylases [184]. Ski and SnoN proteins are absent in normal esophageal epithelium, but strongly expressed in BE epithelium. Upon disease progression, Ski and SnoN expression decreases in low-grade dysplasia and becomes absent in EAC [185]. Their high expression in BE may indicate that they are involved in aberrant TGF-β signalling in this disease.

## 1.17 Protein-protein interactions (PPIs)

A useful way to gain insight into the function of a protein of interest is to study other proteins that it interacts with. PPIs form the basis of cellular and biological processes and signal transduction. Several methods have been developed in the past two decades for identification of protein partners [186, 187]. DNA-binding transcription factors recruit transcriptional cofactors that may act by opening up the chromatin, assisting in recruitment of RNA polymerase, or altering the chromatin structure via histone modifications. The cofactor proteins can be coactivators or corepressors that function in conjunction with the known transcription factor. The role of CDX factors in intestinal differentiation and their aberrant expression levels in IM are well established. However, little is known about the exact molecular mechanisms by which CDXs exert their function as transcriptional activators or repressors. Thus, I carried out large-scale screening to identify novel protein-interacting partners of CDXs that may mediate or modulate their transcriptional activity. A previously described coactivator of CDX2 is p300, which is involved in the transcriptional regulation of the *proglucagon* gene [188].

### *1.17.1 Far western (FW) screening*

FW screening has been successfully used for cloning several important protein molecules that interact with the proteins of interest: Proteins that interact with BRCA1 [189], RB-binding partners such as the retinoblastoma binding protein-1 (RBP-1) [190], and the c-Myc interacting partner Max [191].

In FW screening, a cDNA expression library is first constructed from mRNA of tissue(s) of interest. The library is then ligated to the lambda phage vectors, which are used to transduce host bacteria. After this, the phage-containing bacteria are plated on screening dishes, and protein expression is induced. The induced proteins are immobilized on the solid surface support, typically transferring them onto nitrocellulose filters. The membranes are then blocked to prevent non-specific background, probed with the protein of interest, and finally washed to remove

unbound proteins. The most commonly used baits are *in vitro* translated proteins radioactively labelled with $^{35}S$, so that the positive signals can be detected by exposing on films.

Compared with the two-hybrid system, the advantages of far western blotting are that it can use untagged bait and target proteins and it generally produces few false-positive results. Its disadvantages are the lack of eukaryotic modifications of target proteins, the use of radioactive reagents, potential lack of eukaryotic protein folding, and that only binary complexes can be detected, similarly to the yeast two-hybrid screening. The success of the FW screening depends on the quality of the bait and the representation (amplified or not) of the bacteriophage cDNA library. The strength of interaction between bait and prey ultimately determines the outcome.

### *1.17.2 Yeast two-hybrid (YTH) screening*

YTH screening was first described in 1989 [192] by Fields and Song. It is an *in vivo* genetic assay, which is taking place in the nuclei of the yeast. The assay is based on the bait protein being fused to the DNA-binding domain of the transcription factor Gal4 or LexA, and the prey proteins are fused to the strong transactivation domain of a transcription factor. Only when DNA-binding and transactivating domain are physically reconstituted, the activation of a downstream reporter gene is detected either by growth under selective medium or a color signal will take place [193, 194]. Host organisms have also been expanded to bacteria [195] and mammalian cells [196, 197]. One of the disadvantages of the two-hybrid screening is the high percentage of false positives. Also, false negative interactions resulting from improper protein folding, and lack of mammalian post-translational modifications may pose problems [198, 199]. Lack of physiological relevance may be an issue, because the PPI identified does not happen in the real biological setting, and this requires follow up experiments to validate the true interaction. However, this is a problem presented also in other screening methods. The advantage of YTH is that it is very sensitive and can thus also detect transient interactions.

### *1.17.3 Protein microarrays*

In comparison to the traditional approach, protein microarrays have revolutionized the speed of discovery on a larger scale. It is a relatively new technology offering a platform for diagnostic and prognostic biomarker discovery, and protein interactome studies of transcription factors. The reaction takes place on a protein chip contains up to 9000 full-length and active proteins that represent a wide range of kinases, nuclear receptors, phosphatases, membrane proteins, transcription factors, that are immobilized on nitrocellulose coated glass microscope slides. Bound proteins can be detected via probes that are labelled with fluorescent, affinity, photochemical, or radioisotope tags [200, 201].

### *1.17.4 Co-immunoprecipation (co-IP)*

The process of IP works by adding a specific antibody to a known protein to cell lysates or mixture of proteins. Thus, the antigen can be precipitated , and any proteins that bound to the antibody is washed and eluted. Then, the proteins can be separated on SDS-PAGE and studied further by western blot or by protein sequencing. The advantage of this approach is that protein interaction partners are not limited to binary bindings, but more complex protein complexes can be identified.

# 2 AIMS OF THE STUDY

The present research was centered on the intestine-specific transcription factors caudal-type homeobox proteins CDX1 and CDX2, which function to maintain intestinal phenotype, differentiation, and proliferation. I investigated the regulation of the promoter regions of genes encoding intestinal BA transporters, ASBT and OSTα/β. They are components of the enterohepatic circulation for bile acids, and are found expressed in the terminal ileum. ASBT and OSTα/β are crucial for BA uptake and efflux in enterocytes, respectively. Their transcriptional regulatory mechanisms of genes encoding them have been previously studied, but it was not known, whether the intestine-specific transcription factors CDX1 and CDX2 play additional roles. To emphasize the pathophysiological relevance of this regulation in a disease setting, recent evidence has shown that expression of three BA transporters - ASBT, MRP3, and IBABP - is elevated in esophageal epithelial cells from patients with BE, further emphasizing the relevance of this regulation in a disease setting. However, the precise mechanisms that mediate the aberrant expression of the *ASBT* transporter gene in BE were not known. The following questions were addressed:

- What are the transcriptional regulatory mechanisms of the *ASBT* promoter regulation with regard to CDX1 and CDX2 in cultured cell lines and human ileal and esophageal biopsy specimens?

In addition to the known transcriptional regulatory mechanisms described above for the *OSTα/β* genes, we hypothesized that tissue co-expression of the intestinal transcription factors CDX1 and CDX2 may also play a role in the regulation of OSTα/β expression. The aim of the second part of the present work was to investigate the detailed transcriptional regulation of the *OSTα/β* genes with regard to CDX1 and CDX2, and also whether OSTα/β are detectable in BE biopsies.

- What are the transcriptional regulatory mechanisms of the *OSTα/β* promoters with regard to CDX1 and CDX2 in cultured cell lines and esophageal biopsy tissue?

Another focus of the presented work was to identify novel co-activators and co-repressors of CDX1 and CDX2 by Far Western screening.

# 3 MATERIALS AND METHODS

## 3.1 Chemicals

Deoxyadenosine 5′-[α-$^{32}$P]-triphosphate (6,000 Ci/mmol) and EasyTag™ EXPRESS$^{35}$S Protein Labeling Mix, [$^{35}$S]-,7mCi (259MBq) were purchased from Perkin Elmer (Schwerzenbach, Switzerland). Restriction enzymes were from Roche Diagnostics (Rotkreuz, Switzerland), and PuReTaq Ready-To-Go PCR beads from GE Healthcare (Glattbrugg, Switzerland). The oligonucleotides (Appendix, Table 1) were synthesized by Microsynth (Balgach, Switzerland). SMART cDNA Library Construction Kit was from Clontech (Mountain view, CA, USA). Other chemicals were purchased from Sigma-Aldrich (Buchs, Switzerland), unless stated otherwise.

## 3.2 Cell culture

All cell lines were obtained from (LGC Promochem, Molsheim, France), unless otherwise stated. Human esophagus-derived cell line Het-1A was cultured in Bronchial Epithelial Cell Growth Medium (BEGM) supplemented with SingleQuot kit (Lonza, Basel, Switzerland). Human esophageal OE19 cells were cultured in RPMI-1640 + 2 mM L-glutamine. Human colon-derived T84 cells were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F-12; Invitrogen, Basel, Switzerland), Caco-2 cells in DMEM (Sigma-Aldrich), and LS174T cells in Minimal Essential Medium (MEM; Sigma-Aldrich). DLD1 cells were cultured in RPMI-1640 (Invitrogen). All media for cell lines, except Het-1A, were supplemented with 10 % fetal bovine serum (FBS; Brunschwig, Basel, Switzerland), and 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen). The epithelial cell line IEC-4.1 [202] (a kind gift from Dr. Anthony Jevnikar, Canada), derived from small intestines of BALB/c mice, was cultured on collagen-coated plates in DMEM/F-12 (1:1) supplemented with 5 % FBS, 1 mM sodium pyruvate, 10 ng/ml epidermal growth factor (EGF), and antibiotics as above. All cells were cultured in a humidified atmosphere containing 5 % $CO_2$ at 37°C.

## 3.3 Transfection of cells with siRNAs or plasmids, followed by RNA isolation

Cells were seeded at $10^6$ per well in six-well plates, and transfected with hCDX1 SiGenome SMARTpool (Dharmacon, Lafayette, CO, USA), hCDX2 SiGenome SMARTpool, or siCONTROL non-targeting siRNA #2 (Dharmacon), at a final concentration of 50 nM using the TransIT-TKO reagent (Mirus Bio LLC, LabForce, Switzerland). The transfections were repeated after 24 h, and the cells were harvested in 1 ml TRIzol (Invitrogen) 24 h after the second transfection. To study the effect of CDX overexpression on endogenous ASBT mRNA expression, OE19 cells were seeded on 12-well plates at the density of $2.5x10^5$ cells/well the day before transfections. Cells were transfected with 2.5 μg of either pcDNA3.1 vector control or 2.5 μg of either CDX expression construct, using 3 μl of the Fugene HD transfection reagent (Roche Diagnostics) per microgram of plasmid DNA. Twenty-four hours later, cells were harvested in 0.5 ml of TRIzol/well, and RNAs were processed as described above.

## 3.4 Reverse transcription, and real-time PCR

Four micrograms of RNA isolated with TRIzol reagent were reverse transcribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Rotkreuz, Switzerland), before the real-time PCR analysis using the TaqMan assays Hs00156451_m1 (CDX1), Hs00230919_m1 (CDX2), Hs00166561_m1 (ASBT), Hs00380895_m1 (OST-α), Hs00418306_m1 (OST-β), Hs00167041_m1 (HNF-1α), Hs00200229_m1 (villin), or human ACTB (β-actin) endogenous control (Applied Biosystems), on a 7900HT Fast Real-Time PCR system (Applied Biosystems). The relative mRNA levels were calculated by the comparative threshold cycle method. Within an experiment, all tests were performed in triplicates.

## 3.5 Reporter gene constructs and expression vectors

The human CDX1 and CDX2 expression constructs (kind gifts from Dr. Juan Lucio Iovanna, France) in the pcDNA3.1 vector (Invitrogen) were created as described previously [118]. Mutations in the constructs were corrected to wild-type by site-directed

mutagenesis (QuikChange; Stratagene, La Jolla, CA, USA). Cloning of the human *ASBT* (-1688/+525) [45] and *ASBT* (-830/+525) [44] promoter constructs has been described previously. Cloning of the human *OST*α (-1475/+161) and *OST*β (-4748/+29) promoter reporter constructs has been described previously [18]. The human *ASBT* promoter fragment -99/+525 was amplified using oligonucleotides shown in Table 1, with the promoter construct *ASBT* (-1688/+525) as the template. The PCR fragment was cloned into the pGEM-T vector (Promega, Dübendorf, Switzerland), and further subcloned into the pGL3basic vector (Promega). Point mutations were introduced into the *ASBT* (-830/+525) construct construct using the QuikChange site-directed mutagenesis kit (Stratagene, Basel, Switzerland) and oligonucleotides listed in Appendix Table 1. The mouse *Asbt* promoter fragment (-1810/+3) and the rat *Asbt* promoter fragment (-2975/+147) were cloned from mouse or rat, respectively, genomic DNA (Clontech), using the PCR oligonucleotides shown in Appendix Table 1. Both PCR products were cloned into the pGEM-T vector, and further subcloned into the pGL3basic vector. To create the pTNT-CDX constructs for *in vitro* translation, the CDX1 and CDX2 cDNA fragments were cut out from the pcDNA3.1 constructs with *Eco*RI, and subcloned into the *Eco*RI restriction site of the expression vector pTNT (Promega). The identities of all PCR-cloned fragments were verified by DNA sequencing (Microsynth).

## 3.6 Transient plasmid transfections and luciferase reporter assays

For reporter assays, cells were seeded on 48-well plates at a density of $10^5$/well for Caco-2, LS174T and T84 cells, or 50,000 cells/well for Het-1A, OE19, and IEC-4.1 cells, 24 h before transfection. Cells were cotransfected with 400 ng of the luciferase reporter constructs and 200 ng of the expression plasmids at a ratio of 3 μl FuGENE HD per μg DNA. To normalize the amount of DNA transfected, the pcDNA3.1(+) vector was added when appropriate. To control for transfection efficiency, 100 ng of the Renilla luciferase (pRL-CMV) reporter plasmid (Promega) were cotransfected. Cells were harvested 36 h after transfection, and luciferase activities determined using the Dual Luciferase Assay System (Promega) and a Luminoskan Ascent microplate luminometer (Thermo Fisher Scientific, Wohlen, Switzerland). Reporter activities obtained for the empty pGL3basic corresponding to each test condition, as well as for the test construct containing the test

promoter in the control conditions, were set to 1, and fold activities are shown relative to this. All experimental conditions were performed in triplicate and the experiments were repeated at least three times.

## 3.7 Electrophoretic mobility shift assay (EMSA)

Oligonucleotides used in EMSAs (Appendix, Table 1) were designed to have a 5′-AGCT overhang in the top strand and a 5′-GATC overhang in the bottom strand when annealed, allowing radioactive labelling by fill-in reactions. Fifty nanograms of annealed oligonucleotides were labeled in 20 µl reactions containing 1 µl MultiScribe Reverse Transcriptase (50 U/µl) (Applied Biosystems), 1 µl 1× RT Buffer, 250 nM dGTP/dCTP/dTTP, and 1 µl [α-$^{32}$P]-dATP. Unincorporated nucleotides were removed using Microspin G-25 columns (GE Healthcare). Six micrograms of LS174T nuclear extracts prepared using the NE-PER extraction kit (Pierce, Lausanne, Switzerland) or 1.5 µl of *in vitro* translated CDX1/CDX2 proteins each, generated with TNT T7 Coupled Reticulocyte Lysate System (Promega), using the plasmids pTNT-CDX1 and pTNT-CDX2 as templates, were used for each DNA-binding reaction. Protein DNA complexes were formed in the binding buffer [20 mM Tris–HCl, pH 8.0, 60 mM KCl, 2 mM $MgCl_2$, 12% (v/v) glycerol, 0.3 mM dithiothreitol (DTT), 87.5 ng/µl preboiled poly(dI-dC)-poly(dI-dC)] in a total volume of 20 µl for 10 min at 30°C. After this, 50,000 cpm (0.5–1.5 ng) of the radioactive probes was added to the reactions. For competition EMSAs, 10-, 50-, or 100-fold molar excess of unlabelled *ASBT* promoter CDX-binding site-containing oligonucleotides was added immediately before the radioactive probe containing a consensus CDX response element (*CDXRE*). In supershift experiments, 1 µl of the monoclonal CDX1 antibody [98] (a kind gift from Dr. Walter Bodmer and Dr. Carol Chan, UK), polyclonal CDX2 antibody (C6747; Sigma), polyclonal RXRα antibody (sc-774; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or polyclonal PPARα antibody (sc-1985; Santa Cruz Biotechnology) were added to the extracts 1 h before the probe, and incubated at 4°C. Immediately after the binding reactions, the samples were loaded onto pre-electrophoresed 5% (acrylamide/bis 30:1) native acrylamide gels and run at 200 V in

0.5× TBE for 3 h. The gels were fixed in 10 % (v/v) acetic acid for 10 min, dried onto Whatman DE81 paper under vacuum, and exposed to Kodak BioMax MR-1 films at -80°C.

## 3.8 Chromatin immunoprecipitation (ChIP) assays

ChIP assays were carried out as described previously [203]. T84 cells were grown on 10 cm plates to 80% confluence, and the cells were harvested by crosslinking with 1% methanol-free formaldehyde and processed using the ChIP-IT Express kit (Active Motif, Rixensart, Belgium). Shearing of the chromatin was achieved by five 20 s pulses of sonication with 30 s pauses on ice between each pulse, using a Branson Digital Sonifier (Branson Ultrasonics, Danbury, CT, USA) at a power setting of 25 %. For immunoprecipitations, the samples were incubated with 2 µg negative control mouse IgG1 antiserum (Dako Denmark A/S, Glostrup, Denmark), or with 2 µg of one of the following antibodies: anti-CDX1 (CDX1 Ab1), anti-CDX1 (ab24000; Abcam, Cambridge, UK) (CDX1 Ab2), anti-CDX2 (71-83; Sigma) (CDX2 Ab1), anti-CDX2 (MU392A-100; Biogenex, Fremont, CA, USA) (CDX2 Ab2). Two amplicons were assayed for immunoprecipitated samples, using the oligonucleotide primers listed in Table 1, containing either the *ASBT* promoter region (-736/-154) or a region from the first intron of the *ASBT* gene. After the initial denaturation stage at 94°C for 3 min, the PCR cycling conditions were 94°C for 20 s, 60°C for 30 s, and 72°C for 1 min. For the *ASBT* promoter region, 5 µl of each PCR product was removed after 38 cycles, and for the *ASBT* intron fragment, 5 µl of each PCR product was removed after 30 cycles. The *ASBT* promoter and intronic PCR fragments were resolved on 1.5 % agarose gels, containing GelRed gel stain (Chemie Brunschwig AG, Basel, Switzerland).

## 3.9 Human tissue collection and processing

Signed informed consent forms were collected from all human subjects. The study protocol and consent forms were approved by the Ethical Committees of the sample collection sites. A total of 35 patients were included in the study. BE tissue was collected in 2011 at the Department of Gastroenterology, Qingdao Municipal Hospital, China; Department of Gastroenterology, Shanghai Tenth People's Hospital, Tongji University School of

Medicine, Shanghai, China; Division of Gastroenterology, Chinese PLA General Hospital, Beijing, China, and Department of Gastroenterology, General Hospital of Chinese People's Armed Police Forces, Beijing, China. BE was diagnosed endoscopically and histologically on the basis of typical features such as the presence of specialized columnar epithelium and goblet cells. An experienced local pathologist performed histology. Of the 35 patients, 22 (63%) were male and 13 (37%) were female. The mean age ± SD of the cohort was 52.60 ±10.97 years, and the median age was 52 years. Among the patients, 29/35 (83%) had detectable CDX1 mRNA expression, all 35 had detectable CDX2 expression, and 20/35 (57%) had detectable ASBT expression. The BE samples were homogenized by ultrasound. RNAs were isolated using the TRIzol reagent (Invitrogen), and were quantified with a NanoDrop ND-1000 spectrophotometer (Witec AG, Littau, Switzerland). Ileal biopsies were obtained from 48 healthy patients during routine surveillance colonoscopies in the endoscopy unit of the Division of Gastroenterology and Hepatology at the University Hospital Zurich between 2004 and 2008. Of these 48 patients, 17 (35%) were male and 31 (65%) were female. The mean age ± SD of the cohort was 54.3±13.7 years, and the median age was 58.5 years. Ileal samples were collected in RNAlater (Ambion, Austin, TX, USA), and syringed into TRIzol reagent before RNA isolation. RNAs were quantified with a NanoDrop ND-1000 spectrophotometer.

## 3.10 *In silico* analysis of the *ASBT*, *OSTα*, and *OSTβ* promoter regions

I performed an *in silico* analysis on the proximal 850 base pairs of the human *ASBT*, 927 base pairs on *OSTα* and 1453 base pairs on *OSTβ* proximal promoter (NCBI Reference Sequence: NT_009952.14; NT_029928; NW_925884), to identify putative CDX response elements (*CDXRE*s). The consensus core motif for CDX DNA-binding is 5′-$TTTA^{T}/_{C}$ -3′ or 5'-$^{G}/_{A}TAAA$-3′ [86]. *CDXRE*s within the proximal promoter region were identified by MatInspector software [204] and visual inspection.

## 3.11 Immunohistochemistry

For the evaluation of ASBT and CDX protein expression in formalin-fixed paraffin-embedded tissues, standard immunohistochemical assays involving biotin-avidin-linked peroxidase detection were used. Paraffin-embedded sections were dissolved by placing the glass slides on heating blocks for 10 min. Slides were deparaffinized by two 5 min changes of xylene. The sections were dehydrated through a graded series of 80 %, 95 %, and 100 % ethanol, washed three times in PBS, and placed in 0.5 % $H_2O_2$ for 30 min to block endogenous peroxidase activity. Antigen retrieval was performed by immersing slides in 10 mM citrate buffer, pH 6.0, placing them in a pressure cooker until boiling point, and then allowing them to sit for 5 min. Slides were taken out and rinsed three times with PBS at 3 min intervals. Slides were incubated overnight at 4°C with rabbit polyclonal anti-ASBT antibody at a final concentration of 1:400 (a kind gift from Paul Dawson, Wake Forest University Baptist Medical Center, Winston-Salem, NC, USA), anti-CDX2 antibody at a final concentration of 1:200 (Maixin MAB-0216, China), or anti-CDX1 at a final concentration of 1:200 (AP6130a; Abgent, San Diego, CA, USA). Slides were again rinsed with PBS and biotinylated secondary antibodies were added and incubated at room temperature for 1 h. Slides were taken out and rinsed three times with PBS at 3 min intervals and lightly counter-stained with hematoxylin.

## 3.12 Statistical analysis

All cell-based experiments were independently repeated at least three times, and representative experiments are shown. All quantitative data are reported as means ± SDs. Differences between experimental groups were analyzed by one-way analysis of variance (ANOVA) with Tukey's post hoc test. *P* values $<0.05$ were considered significant. For the correlation of mRNA expression levels in human tissue, logarithmic values of mRNA levels were analyzed using linear regression. All statistical analysis was carried out using GraphPad Prism software (GraphPad, San Diego, CA, USA).

## 3.13 Recipes of media and buffers used in far western screening

- 1 M $MgSO_4$ — 12.02 g/100 ml $H_2O$ (Filter-sterilized)
- 10× Lambda dilution buffer — 1 M NaCl<br>0.1 M $MgSO_4.7H_2O$<br>0.35 M Tris–HCl, pH 7.5
- 1× Lambda dilution buffer — dilute the stock from above 1:10<br>0.01 % gelatin
- $MgSO_4$ plates — LB agar +10 mM $MgSO_4$
- LB/ $MgSO_4$ soft top agar (1L) — 10 mM $MgSO_4$ (10mM final conc.)<br>7.2 g agarose
- IPTG 10 mM in $H_2O$
- XL1-blue growth medium — LB medium<br>10 mM $MgSO_4$<br>0.2 % maltose<br>15 μg/ml tetracycline
- Pre-probing buffer — 1× binding buffer<br>0.3 mM DTT<br>0.05 mg/ml human placental DNA (boiled at 98°C for 4-5min and cooled to room temperature)<br>Complete protease inhibitors (Roche Diagnostics): 1 tablet/200 ml
- 5× binding buffer — 100 mM Tris–HCl, pH 8.0<br>300 mM KCl<br>10 mM $MgCl_2$<br>60 % glycerol

## 3.14 Preparation of $^{35}$S-labelled proteins

*In vitro* translation of CDX1 and CDX2 proteins was carried out using TNT Quick Coupled Transcription/Translation Systems (Promega, USA) with the following components: 500 µl TNT Rabbit Reticulocyte Lysate, 40 µl TNT Reaction Buffer, 20 µl TNT RNA Polymerase (T7), 20 µl 1 mM Amino Acid Mixture Minus Cysteine and Methionine, 80 µl Promix [L]-$^{35}$S-Met/Cys, 20 µl (40 U/µl) RNasin Ribonuclease inhibitor, 40 µl (1 µg) DNA templates, and 280 µl of nuclease-free water to a final volume of 1000 µl. The reaction was incubated at 30°C for 90 min.

## 3.15 Purification of the *in vitro* translated proteins

To remove excess radioactive amino acids and thus to reduce the background after film exposure, Microcon centrifugal filter units (Millipore, Billerica, MA, USA), with a protein cutoff size of 10 kDa, were used. Therefore, any molecules smaller than 10 kDa were removed. Two hundred microliters of CDX1 or CDX2 proteins were mixed with 200 µl of far western binding solution, and loaded on the Microcon spin column at a speed of 13,000 *g* for 1 h. The washing step with FW binding solution was repeated three times.

## 3.16 Protein identity confirmation

Protein concentrations of purified samples were determined with the BCA protein assay reagent (Thermo Scientific). Two microliters of proteins from purified *in vitro* translated CDX1 and CDX2 protein samples were mixed with 8 µl PBS. Proteins were separated on 10 % SDS-polyacryamide gels, which were then fixed in the following solutions: 50 % methanol + 10 % acetic acid for 15 min, 10 % methanol + 5 % acetic acid for 10 min, and 10 % glycerol for 5 min. The gels were then dried onto Whatman 3MM paper. After exposure to films, actual protein sizes were compared to the predicted protein sizes of CDX1 and CDX2.

## 3.17 cDNA library construction from human small intestine and colon

*(all procedures described in this section are modified based on the Clontech Laboratories cDNA library construction kit user manual)*

### *3.17.1 First-strand cDNA synthesis*

The following reagents were combined in a sterile 0.5 ml microcentrifuge tube: 0.5 µl small intestine poly A + RNA, 0.5 µl colon poly A + RNA (Clontech), 1 µl SMART IV Oligonucleotide, 1 µl CDS III/3′ PCR Primer, and 2 µl water to a final volume of 5 µl. The contents were mixed and incubated at 72°C for 2 min, and cooled on ice for 2 min. The following were added to each reaction tube: 2 µl 5× First-Strand Buffer, 1 µl 20 mM DTT, 1 µl 10 mM dNTP Mix, and 1 µl MMLV Reverse Transcriptase to a final volume of 10 µl. The contents of the tube were mixed by gentle pipetting and briefly centrifuged to bring the contents down. The tubes were then incubated at 42°C for 1 h, and placed on ice to terminate first-strand synthesis.

### *3.17.2 cDNA amplification by long-distance PCR*

The following components were combined in the reaction tube: 1 µl First-Strand cDNA, 40 µl deionized water, 5 µl 10× Advantage 2 PCR Buffer, 1 µl 50X dNTP Mix, 1 µl 5′ PCR Primer, 1 µl CDS III/3′ PCR Primer, and 1 µl 50× Advantage 2 Polymerase; topped up with water to a final volume of 50 µl. The contents were briefly mixed and amplified with the following program 1 cycle of 95°C for 1 min, 20 cycles of 95°C for 15 s and 68°C for 6 min.

### *3.17.3 Proteinase K digestion*

Fifty microliters of amplified double-stranded cDNA (2-3 µg) and 2 µl proteinase K (20 µg/µl) were added to a sterile 0.5 ml tube. The sample was mixed and centrifuged briefly to bring the contents down, and incubated at 45°C for 20 min. Fifty microliters of deionized water was added to the tube. One hundred microliters of phenol:chloroform:isoamyl alcohol were added and mixed by continuous gentle inversion

for 1-2 min. The tube was centrifuged at 14,000 rpm for 5 min to separate the phases. The top phase (aqueous) layer was moved to a clean 0.5 ml tube, and the interface and lower layers were discarded. One hundred microliters of chloroform:isoamyl alcohol was added to the aqueous layer, which was mixed by continuous gentle inversion for 1-2 min, followed by centrifugation at 14,000 rpm for 5 min to separate the phases. The top (aqueous) layer was moved to a clean 0.5 ml tube, and the interface and lower layers were discarded. Ten microliters of 3 M sodium acetate, 1.3 µl glycogen (20 µg/µl) and 260 µl 95% ethanol at room temperature were added, followed by centrifugation at 14,000 rpm for 20 min at room temperature.

### *3.17.4 SfiI digestion*

The following were mixed in a tube and incubated at 50°C for 2 h: 79 µl cDNA, 10 µl 10× SIFI Buffer, 10 µl *Sfi*I restriction enzyme, and 1 µl 100× bovine serum albumin. The incubation was followed by addition of 2 µl 1% xylene cyanol dye (0.1 g in 10 ml water).

### *3.17.5 cDNA size fractionation by CHROMA SPIN-400*

Sixteen 1.5 ml tubes were labelled and arranged in a rack in order. To prepare the CHROMA SPIN-400 Column for the drip procedure, the columns were inverted several times to resuspend the gel matrix completely and to remove any air bubbles. A 1 ml pipettor was used to resuspend the matrix gently, avoiding generating air bubbles. The bottom cap was removed and the column was allowed to drip by gravity. The storage buffer drained through the column by gravity flow until I could see the surface of the gel beads in the column matrix. The top of the column matrix should be at the 1.0 ml mark on the wall of the column. The flow rate should be approximately 1 drop/40-60 s. The volume of one drop should be approximately 40 µl. If the flow rate was too slow (i.e., more than 1 drop/100 s) and the volume of one drop was too small (i.e., <25 µl), the matrix should be resuspended completely and the drip procedure repeated until it reached the above parameters.

When the storage buffer stopped had dripped out, I carefully added (along the column inner wall) 700 µl column buffer to the top of the column and allowed it to drain out. When this buffer stopped dripping (after 15-20 min), I carefully and evenly applied ~100 µl mixture of *Sfi*I-digested cDNA and xylene cyanol dye to the top-central surface of the matrix. Before proceeding to the next step, the sample was allowed to be fully absorbed into the surface of the matrix. With 100 µl column buffer, I washed the tube that contained the cDNA, and gently applied this material to the surface of the matrix. I allowed the buffer to drain out of the column until there was no liquid left above the resin. When the dripping ceased, I proceeded to the next step. At this point, the dye layer should be several millimeters into the column. I placed the rack containing the collection tubes under the column, so that the first tube was directly under the column outlet. The total of sixteen drops (fractions) was collected. To this end, I added 600 µl column buffer and immediately began collecting single-drop fractions (~35 µl per tube) in tubes 1–16. Each tube was capped after each fraction was collected, and the column was re-capped after fraction 16 had been collected.

The following reagents were added to the tube with 3-4 pooled fractions with size fractions between 1-4 kb (judged by agarose gel analysis) containing the cDNA, 1/10 volume sodium acetate (3 M; pH 4.8), 1.3 µl glycogen (20 mg/ml), and 2.5 volumes of 95 % ethanol (-20°C). The tube was placed at -20°C overnight for better precipitation and recovery of cDNA. The tube was then centrifuged at 14,000 rpm for 20 min at room temperature. The supernatant was removed with a pipette. The tube was briefly centrifuged to bring all the remaining liquid to the bottom. All liquid was removed and the pellet was allowed to air dry for ~10 min, and resuspended in 7 µl deionized water and mixed gently. The *Sfi*I-digested cDNA was then ready to be ligated to the *Sfi*I-digested, dephosphorylated λTriplEx2 Vector.

### *3.17.6 Ligation of cDNA to lambda λTriplEx2 vector*

The following components were combined in a tube: 1.5 µl cDNA library, 1 µl vector (500 ng/µl), 0.5 µl 10× ligation buffer, 0.5 µl 10 mM ATP, 0.5 µl T4 DNA ligase, and 1 µl deionized water in a final volume of 5 µl. The mixture was incubated at 16°C overnight.

### *3.17.7 Package of ligation to the lambda phage*

The Gigapack III Packaging Extracts (Agilent Technologies, Santa Clara, CA, USA) was quickly thawed by holding the tube between the fingers. One microliter of cDNA was added immediately to the packaging extract. The contents were mixed by pipette and incubated at room temperature for 2 h. Five hundred microliters of SM buffer was added to the tube. The tube was centrifuged briefly to sediment the debris, and 20 µl chloroform was added. At this stage, the phage could be used for titration.

### *3.17.8 Titration of the unamplified library*

Determination of the titer (e.g., plaque-forming units (pfu)/µl) of the unamplified library gives an estimate of the number of independent phage clones in the library. A single isolated colony from the working stock plate (XL1-Blue) was used to inoculate 15 ml LB/$MgSO_4$/maltose broth in a 50 ml test tube or an Erlenmeyer flask. The flask was incubated at 37°C overnight while shaking (at 140 rpm) until the optical density ($OD_{600}$) of the culture reached 2.0. The cells were centrifuged at 5,000 rpm for 5 min. The supernatant was poured off, and the pellet was resuspended in 7.5 ml 10 mM $MgSO_4$. Appropriate dilutions of each of the packaging extracts were made in 1× lambda dilution buffer. As a general guideline, an appropriate dilution for an unamplified λ lysate is 1:5 to 1:20. One microliter of the diluted phage was added to 200 µl XL1-Blue overnight culture, and the phages were allowed to adsorb at 37°C for 10-15 min. Two milliliters of melted LB/$MgSO_4$ top agar were added and mixed by rapid inversion, before being immediately poured onto 90 mm LB/$MgSO_4$ plates pre-warmed to 37°C. The plates were swirled quickly after pouring to allow even distribution of the top agar, cooled at room temperature for 10 min to allow the top agar to harden, inverted, and incubated at 37°C for 6-18 h. The plates were checked periodically to ensure that plaques were developing. The plaques were counted and the phage titer was calculated using the following formula:

$$\text{pfu/ml} = \frac{\text{number of plaques x dilution factor x } 10^3 \text{ µl/ml}}{\text{µl of diluted phage plated}}$$

### *3.17.9 Determining the percentage of recombinant clones*

To perform blue/white screening in *E. coli* XL1-Blue, the procedure above for titering an unamplified library on LB/$MgSO_4$ plates was followed, except IPTG and X-gal were added to the melted top agar before plating the phage/bacteria mixtures. For every 4 ml of melted top agar, I used 40 µl each of the 100 mM IPTG and 100 mM X-gal solutions, and aimed for 500-1,000 plaques/90 mm plate. Plates were incubated at 37°C for 6-18 h, or until plaques and blue colour developed. The ratio of white (recombinant) to blue (non-recombinant) plaques gave an estimate of recombination efficiency. Successful ligation using cDNA synthesized from control poly $A^+$ RNA resulted in at least 80 % recombinants.

### *3.17.10 cDNA library amplification*

A single isolated colony was selected from the primary working plate of XL1-Blue, and used to inoculate 15 ml LB/$MgSO_4$ maltose broth, which was incubated at 37°C overnight with shaking (140 rpm). The cells were centrifuged for 5 min at 5,000 rpm, the supernatant was poured off, and the pellet was resuspended in 7.5 ml 10 mM $MgSO_4$. The required number of LB/$MgSO_4$ agar plates was established, along with the required number of 4 ml tubes with 500 µl overnight bacterial cultures and enough diluted lysate to yield 6–7 × $10^4$ plaques per 150 mm plate. The tubes were incubated in a 37°C water bath for 15 min, and 4.5 ml melted LB/$MgSO_4$ soft-top agar was added to each tube. The phage/bacteria preparation was mixed and poured onto LB/$MgSO_4$ agar plates. The plates were swirled quickly, while pouring, to promote even distribution of the melted agar over the plates. The plates were cooled at room temperature for 10 min to allow the top agar to harden. The plates were inverted and incubated at 37°C for 6-18 h, or until the plaques become near confluent (i.e., touching each other). Twelve milliliters of 1× lambda dilution buffer was added to each plate, which was then stored at 4°C overnight. The eluted phages were then ready to be pooled in 1X lambda dilution buffer to form an amplified library lysate. To this end, the plates were incubated at room temperature for 1 h on a platform shaker (~50 rpm), after which the λ-phage lysates were poured into a sterile beaker. This formed the pooled, amplified library lysate. To clear the phage lysate of cell debris and to lyse any remaining intact cells: the phage lysate was mixed well and poured into sterile 50 ml glass screw-cap tubes, and 10 ml chloroform was added to the pooled lysates. The caps were screwed on

tightly and the tubes vortexed for 2 min. The tubes were then centrifuged in a Beckman J2-21 centrifuge at 7,000 rpm (5000 *g*) for 10 min. The supernatant was collected in new sterile 5 ml glass tubes, the caps were tightened, and the tubes were stored at 4°C until further use.

### *3.17.11 Titration of the amplified library*

To titer the amplified library, I repeated the procedures described for titration of the unamplified library (Section 3.17.10). A single isolated colony was selected from the working stock plate of XL1-Blue and used to inoculate 20 ml LB/$MgSO_4$/maltose broth (without antibiotics). The culture was incubated at 37°C overnight with shaking (140 rpm) until $OD_{600}$ reached 2.0. The cells were centrifuged at 5,000 rpm for 5 min, the supernatant was poured off, and the pellet was resuspended in 7.5 ml 10 mM $MgSO_4$. Four LB/$MgSO_4$ agar plates (90 mm) were warmed and dried. To prepare dilutions of phage lysate (library), 10 µl of the library lysate was pipette into 1 ml 1× lambda dilution buffer (Dilution 1 = 1:100); and 10 µl of Dilution 1 was transferred into a second tube containing 1 ml 1× lambda dilution buffer (Dilution 2 = 1:10,000).

### *3.17.12 Primary far western screening*

On the day before screening, *E. coli* XL1-Blue was inoculated into 50 ml medium without tetracycline. The bacteria were pelleted by centrifugation at ~1,500 *g* for 5 min. The pellet was resuspended in 25 ml lambda dilution buffer + 10 mM $MgSO_4$. I took 500 µl of the bacterial solution and added 32,000 pfu of the lambda library, and incubated the bacteria/phage mixture for 15 min at 37°C. I added 10 ml of top agarose to 580 µl bacteria/phage mix poured the resultant mixture onto dry, warmed 15 cm agar/10 mM $MgSO_4$ plates. The top agarose was kept at a maximum temperature of 45°C in a water bath. The top agar was allowed to harden and the plates were incubated at 37°C for 3-5 h until plaques were forming. Hybond-C Extra supported mixed ester nitrocellulose membranes were marked with a ballpoint pen or pencil, and soaked in ~50 ml 10 mM IPTG solution. As soon as plaques began to appear, one soaked Hybond-C Extra membrane was added to each plate avoiding bubbles. The Hybond-C Extra membranes were first pad-dried on filter paper if too wet. The Hybond-C Extra membranes (now on

the plates) were marked using a needle with asymmetrically arranged holes through the membrane and agar. The plates were incubated for 3-4 h and kept at 4°C overnight.

### *3.17.13 Membrane blocking and addition of probes*

Two Hybond-C Extra membranes were removed from the plates and placed on top of each other in a hybridization bag. It was important that the side with the proteins was against the hybridization bag and not the other membrane. The bags were sealed as close to the filters as possible. This decreased the volume of buffer and radioactivity needed. I used 10 ml of pre-probing buffer per two filters in one bag, and incubated for 30 min at 30°C. The radioactive protein probes were then added (~10,000 cpm/ml), and the bags inverted gently to mix the contents. The bags were incubated for 1 h at 30°C while shaking gently, and then emptied by decanting. The membranes were then washed with wash buffer in the bags until the signals on the filters were not notably detected by a Geiger counter. This was followed by two washing steps on plastic trays. The membranes were finally dried on Whatman 3MM chromatography paper overnight.

### *3.17.14 Film exposure*

The dried Hybond-C Extra membranes were exposed on Kodak Biomax MR single films for 2-3 days for optimal signal, before the films were developed.

### *3.17.15 Alignment of blots*

The blots were aligned with the agar plates using the asymmetric holes earlier made with the needle, and the plaques that gave a signal were marked at the bottom of the agar plates. The plaques were cut out using plastic 3ml Pasteur pipettes. The cut agar plugs containing the selected plaques were placed in 500 µl lambda dilution buffer and eluted at 4°C until further use.

### *3.17.16 Secondary screening*

On the day before screening, *E. coli* XL1-Blue was inoculated into 50 ml medium without tetracycline. Bacteria (20 ml) were pelleted by centrifugation at ~1,500 *g* for 5 min, and resuspend in 10 ml lambda dilution buffer + 10 mM $MgSO_4$. Then, aliquots of 200 ul of the bacterial solution were taken, and approximately 40 pfu from the diluted and titrated

eluted phages picked as positives from the primary screen were added, and the mixtures plated on 10 cm plates. The bacteria/phage mixtures were incubated at 37°C for 15 min, after which 5 ml of melted and cooled top agarose were added to the bacteria/phage mixtures. These were then poured onto dry, warmed, 10 cm agar plates containing 10 mM $MgSO_4$, taking care to avoid bubbles. The top agarose was kept at a maximum temperature of 45°C in a water bath. The top agarose was allowed to harden, and the plates were incubated for 3-5 h until plaques started to form. Hybond-C Extra membranes were marked with a ballpoint pen or pencil, and soaked in ~30 ml 10 mM IPTG solution. As soon as plaques began to appear, one soaked Hybond-C Extra membrane was placed on each plate, avoiding bubbles between the membrane and top agarose. The membranes were pad-dried on filter paper if necessary. The Hybond-C Extra membranes (now on the plates) were marked, using needles, with asymmetrically arranged holes through the membrane and agar. The plates were then incubated for 3-4 h, and placed at 4°C for overnight.

### *3.17.17 Film exposure*

See above (3.17.14).

### *3.17.18 Alignment of blots*

See above (3.17.15).

# 4 RESULTS

## 4.1 Regulation of the gene encoding the intestinal bile acid transporter ASBT by the caudal-type homeobox proteins CDX1 and CDX2

### *4.1.1 Expression profiling of genes of interest in human intestinal cell lines*

To investigate the expression profiles of genes of interest in the intestinal cell lines, I analyzed the relative gene expression levels in human colon adenocarcinoma-derived cell lines: HT29, DLD1, Caco-2, T84, and LS174T. Despite the same tissue origin of these cell lines the mRNA expression levels of CDX1, CDX2, HNF-1α, GATA4, and ASBT varied considerably. When analyzing the expression levels of these genes, we found that HNF-1α levels appeared to correlate with ASBT, whereas CDX1 and CDX2 expression levels do not. Thus, we speculate that HNF-1α, but not CDXs, maintains the baseline ASBT expression level (Fig. 4.1).

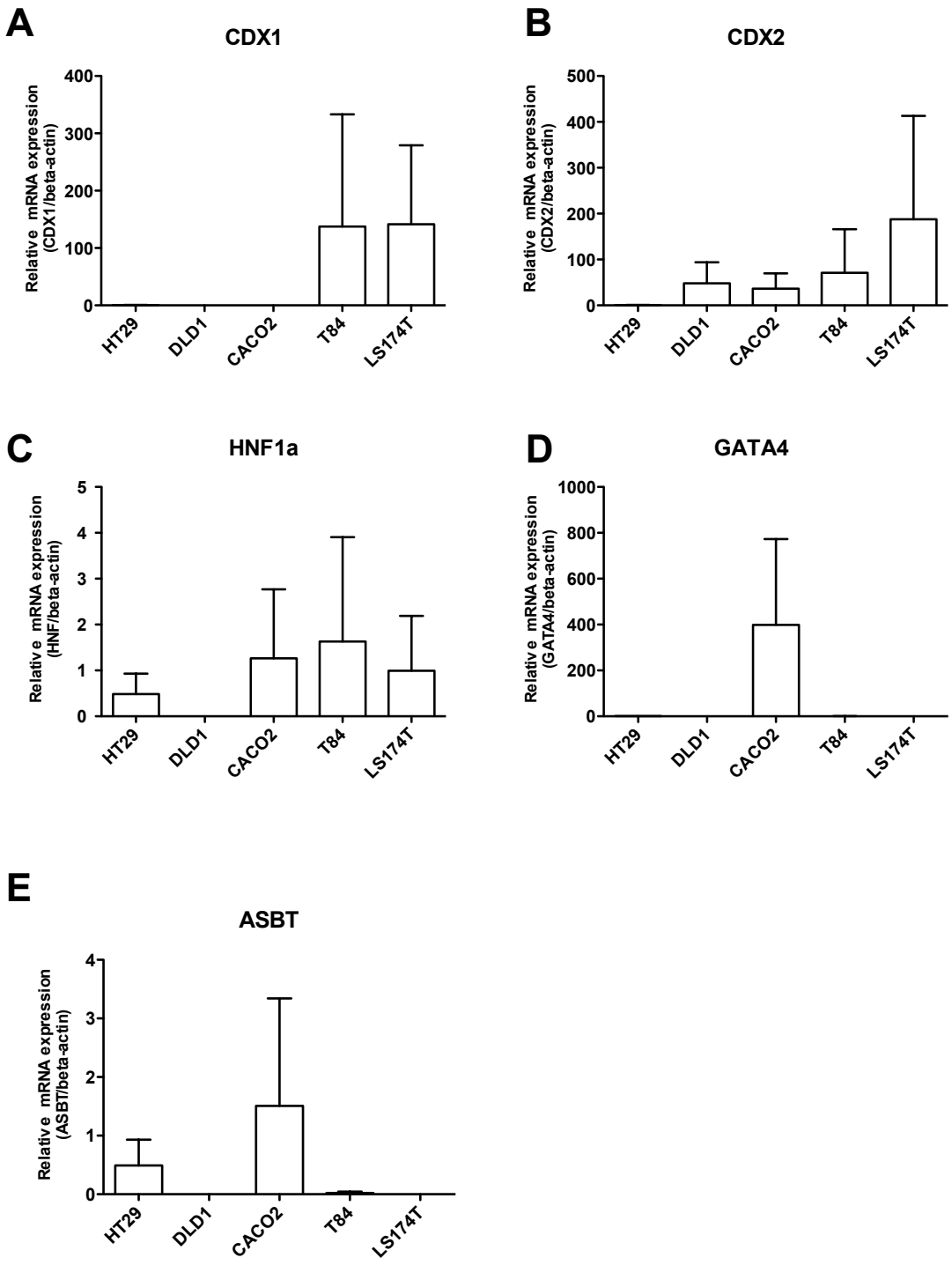



**Fig. 4.1 Endogenous mRNA expression levels in HT29, DLD1, Caco-2, T84, and LS174T cell lines.** *A*, CDX1; *B*, CDX2; *C*, HNF-1α; *D*, GATA4; *E*, ASBT. All gene expression levels were relative to those of β-actin.

### *4.1.2 Modulation of endogenous ASBT mRNA expression levels by CDX knockdown or overexpression*

To investigate whether endogenous human ASBT mRNA levels were regulated by CDX1 and CDX2, I performed knockdown experiments with pools of siRNAs specifically targeting CDXs. For this purpose, I chose the human colon cancer cell line T84, which endogenously expresses CDX1, CDX2, and ASBT, all at detectable levels. By targeting the endogenous CDX1 or CDX2 with specific siRNAs, ASBT mRNA expression was significantly decreased compared to that in control cells (Fig. 4.2*A*). Upon CDX1 siRNA treatment, there was an 80 % loss of endogenous CDX1 mRNA, and no decrease in the endogenous CDX2 mRNA level (Fig. 4.2*B*). Conversely, upon CDX2 siRNA treatment, there was a significant loss of endogenous CDX2 mRNA, but endogenous CDX1 mRNA expression level remained unchanged (Fig. 4.2*C*). The siRNA knockdowns thus worked in a gene-specific manner. In a converse approach, I transiently overexpressed CDX1 and CDX2 in human esophagus-derived OE19 cells. As shown in Fig. 4.2*D*, endogenous mRNA levels of ASBT were significantly increased upon exogenous expression of CDX1 and CDX2 in the esophageal cell line.

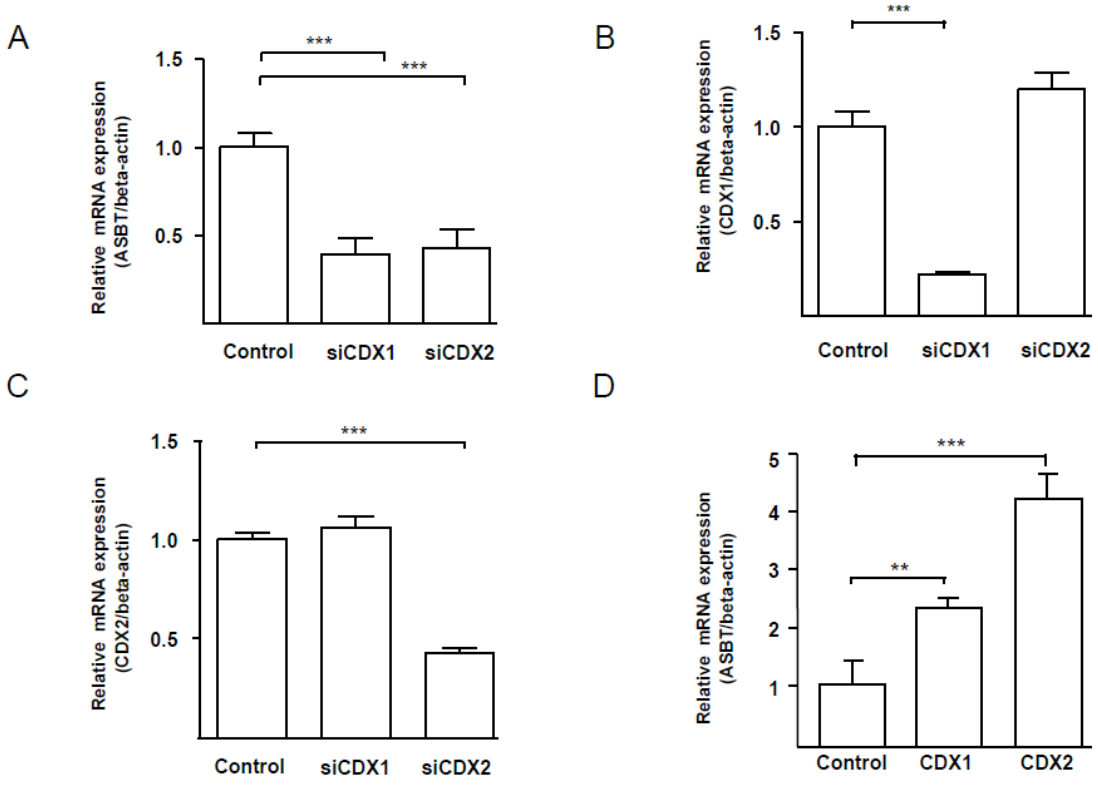
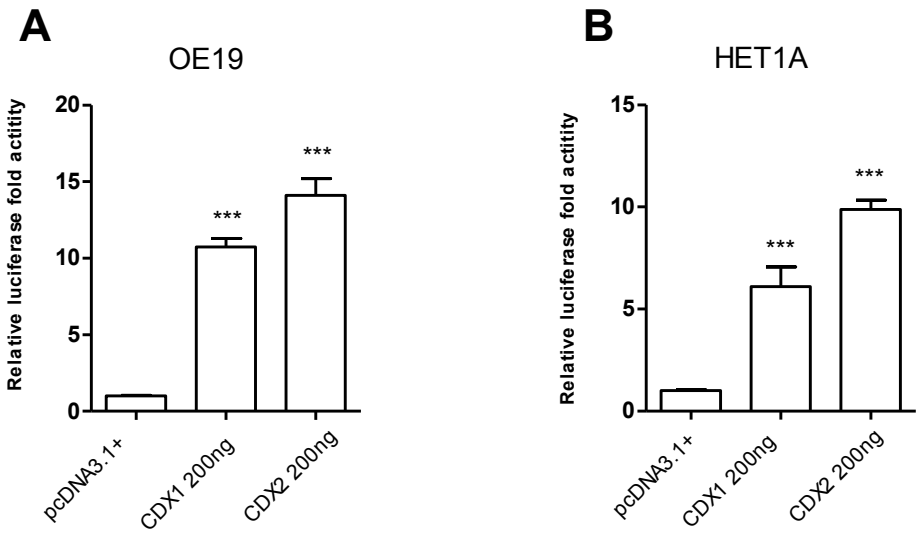



**Fig. 4.2 Modulation of endogenous ASBT mRNA expression levels by CDX1 and CDX2.** *A–C*, ASBT mRNA expression levels were reduced upon knockdown of endogenous CDX1 and CDX2 expression in T84 cells. Cells were transfected with control non-targeting siRNA, siCDX1, or siCDX2 at a final concentration of 50 nM twice at a 24 h interval, and harvested after 48 h. ASBT, CDX1 and CDX2 mRNA expression levels were normalized to those obtained for β-actin. *A*, Knockdown of CDX1 or CDX2 led to a significant decrease in ASBT mRNA expression. *B*, CDX1 siRNAs significantly reduced CDX1, but not CDX2 expression. *C*, CDX1 siRNAs significantly reduced CDX1, but not CDX2 expression. *D*, Exogenous expression of CDX1 and CDX2 significantly increased endogenous ASBT mRNA levels in human esophagus-derived OE19 cells. ***P*<0.01; ****P*<0.001.

### *4.1.3 CDX1 and CDX2 transactivate the ASBT promoter*

To study further the possible direct role of CDX1 and CDX2 in the regulation of the *ASBT* promoter, I transiently transfected two human esophagus-derived cell lines and three human colon-derived cell lines with expression plasmids for human CDX1 and CDX2, together with the *ASBT* (-1688/+525; numbering relative to the transcriptional start site) promoter luciferase construct. Both CDXs increased *ASBT* promoter activity in two esophageal cell lines, OE19 (Fig. 4.3*A*) and Het-1A (Fig. 4.3*B*). Potent CDX-mediated activation of the *ASBT* promoter was also observed in intestinal T84 (Fig. 4.3*C*), Caco-2 (Fig. 4.3*D*), and LS174T (Fig. 4.3*E*) cells. A comparative study with human, mouse and rat *ASBT*/*Asbt* promoter luciferase constructs transfected into Caco-2 and murine IEC-4.1 cells revealed an increased activity of the mouse *Asbt* promoter by both CDX1 and CDX2 in both cell lines, whereas the rat *Asbt* promoter was significantly activated by CDX1 and CDX2 in IEC-4.1 cells, but only by CDX2 in Caco-2 cells (Fig. 4.3*F* and *G*).



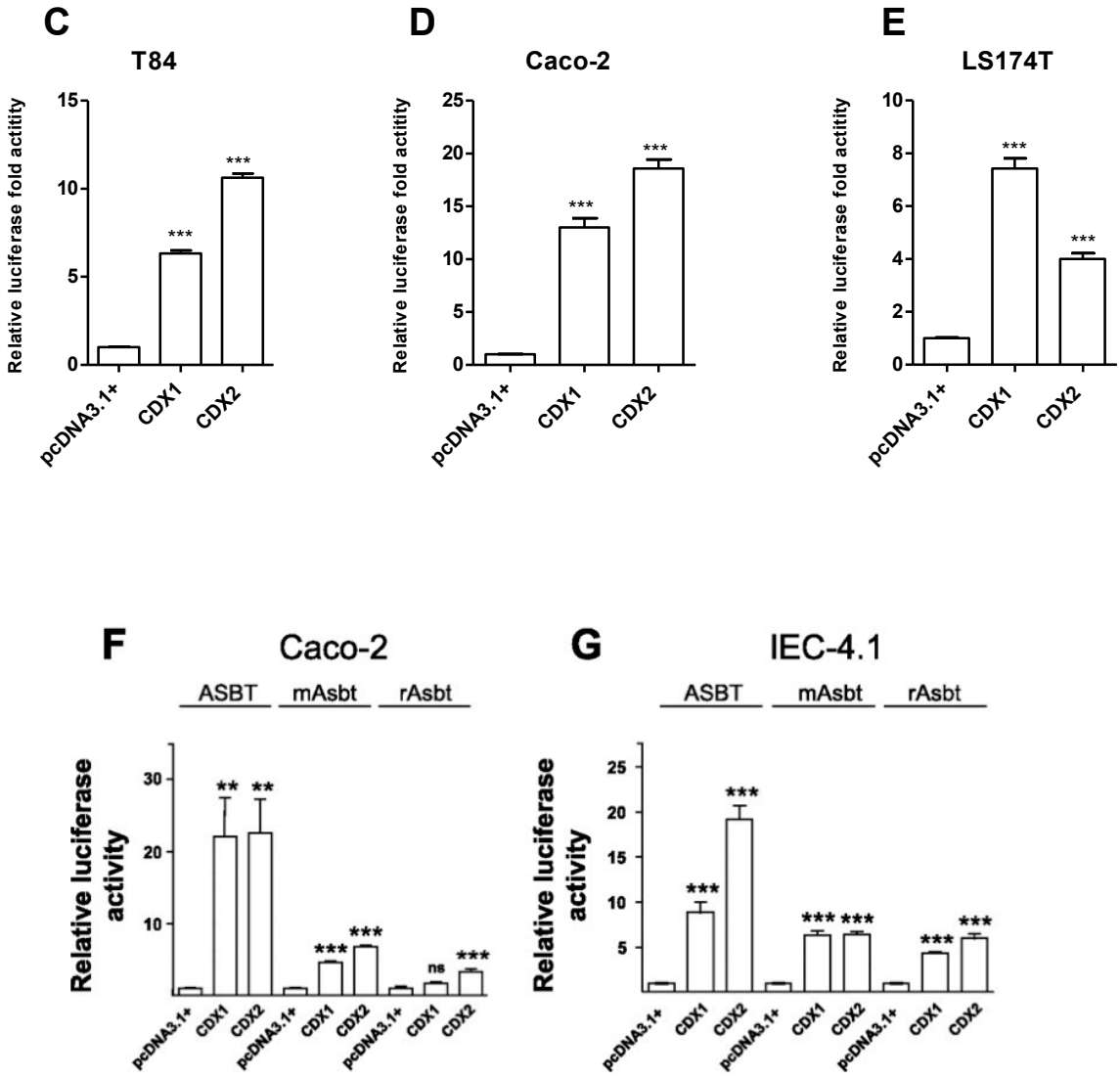



**Fig. 4.3 CDX1 and CDX2 transactivate the human *ASBT* (*A–E*), and mouse (m*Asbt*; *F* and *G*) and rat (r*Asbt*; *F* and *G*) *Asbt* promoters.** Two hundred nanograms of CDX1 or CDX2 expression plasmids were transfected into OE19, Het-1A, T84, Caco-2 and LS174T cells, together with 400 ng of *ASBT* (-1688/+525) promoter luciferase construct. Reporter activities were measured 36 h later. *A*, OE19 cells; *B*, Het-1A cells; *C*, T84 cells; *D*, Caco-2 cells; *E*, LS174T cells; *F*, Caco-2 cells; *G*, IEC-4.1 cells. ***P*<0.01; ****P*<0.001; ns, not significant.

### *4.1.4 In silico analysis of the ASBT promoter region*

I performed an *in silico* analysis of the proximal 850 base pairs of the human proximal promoter and 525 base pairs of the 5'-untranslated (UTR) *ASBT* gene region (NCBI Reference Sequence: *NT_009952.14*), to identify putative *CDXRE*s. The consensus core motif for CDX DNA-binding is 5′-*TTTA*$^{T}/_{C}$-3′ or 5′-$^{G}/_{A}$*TAAA*-3′ [86]. We identified 12 putative *CDXRE*s within the proximal *ASBT* (-850/+525) promoter region by MatInspector software [204] and visual inspection (Fig. 4.4).

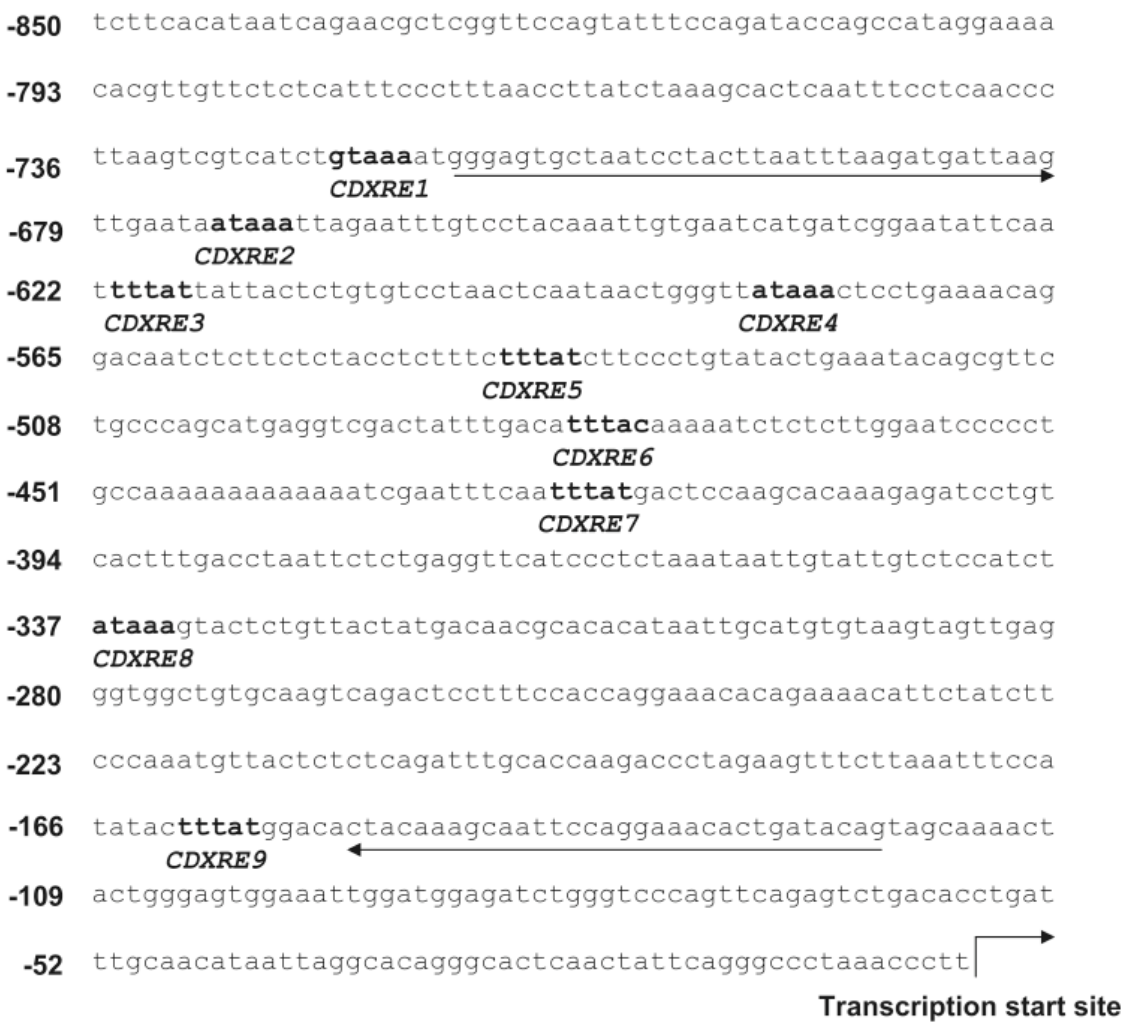



**Fig. 4.4 Sequence of the proximal *ASBT* promoter region (-850/+525).** The transcription start site is indicated by an arrow. The consensus motifs found in the nine predicted *CDXRE*s are shown in bold. The location of the oligonucleotide primers used in ChIP assays are indicated with underlining arrows. The positions of the predicted promoter *CDXRE*s are shown in bold. The location of the oligonucleotide primers used in ChIP assays are indicated with underlining arrows. The positions of the predicted *CDXRE*s were as follows: *CDXRE1* (-722/-717); *CDXRE2* (-672/-667); *CDXRE3* (-621/-616); *CDXRE4* (-583/-578); *CDXRE5* (-541/-536); *CDXRE6* (-480/-475); *CDXRE7* (-424/-419); *CDXRE8* (-337/-332); and *CDXRE9* (-161/-156). We further found three putative *CDXRE*s in the 5'-UTR region of the *ASBT* gene: *CDXRE10* (+274/+278); *CDXRE11* (+303/+307); and *CDXRE12* (+412/+426).

### *4.1.5 The region containing the predicted CDX binding sites mediates ASBT promoter activation*

To study whether the proximal promoter region containing the predicted *CDXRE*s could mediate CDX-dependent activation, I used two *ASBT* promoter deletion constructs in transient transfection of Caco-2 cells. The construct *ASBT* (-830/+525), which contained all the predicted *CDXRE*s, was potently activated by both CDX1 and CDX2, whereas the construct *ASBT* (-99/+525), lacking all nine predicted promoter *CDXRE*s, exhibited significantly reduced activation (Fig. 4.5*A*). This effect was also observed in human EAC-derived cell line OE19 (Fig. 4.5*B*). Although a similar trend was observed in another esophagus-derived cell line Het1A; the loss of activation upon deletion of the *CDXRE*s was not statistically significant (Fig. 4.5*C*). The activation of the *ASBT* promoter by CDXs was not entirely abolished, suggesting that ther *CDXRE*s located in the 5′-UTR of the *ASBT* gene may contribute to the effect.

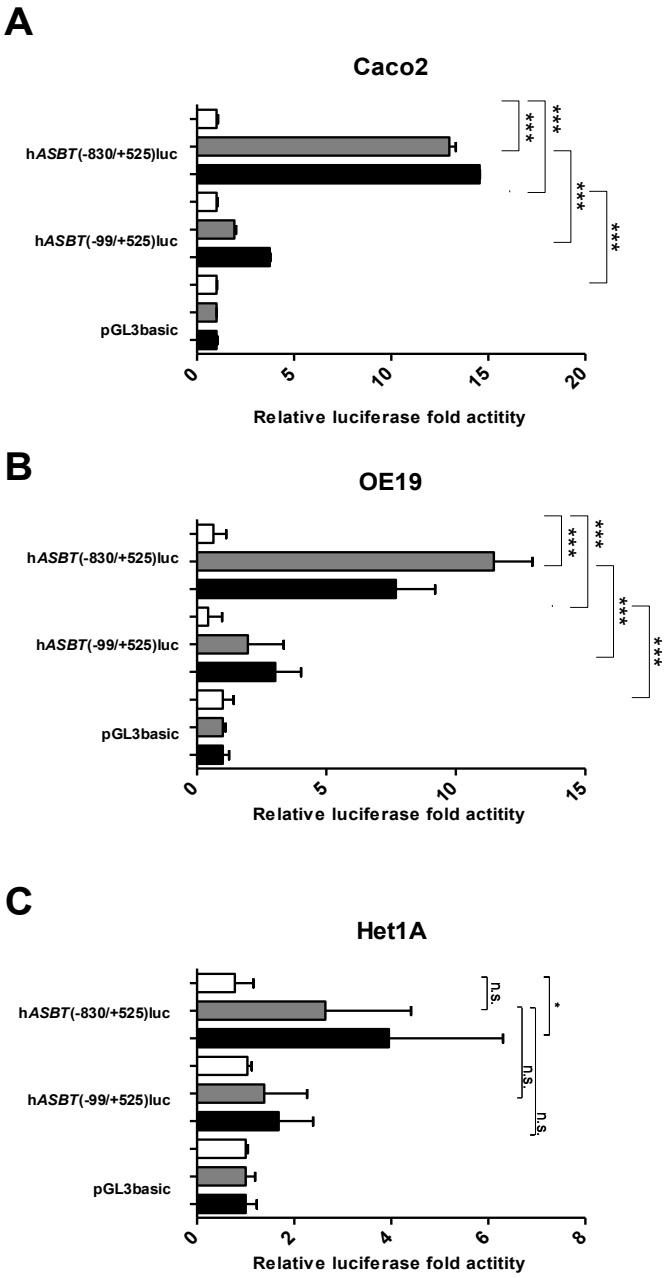
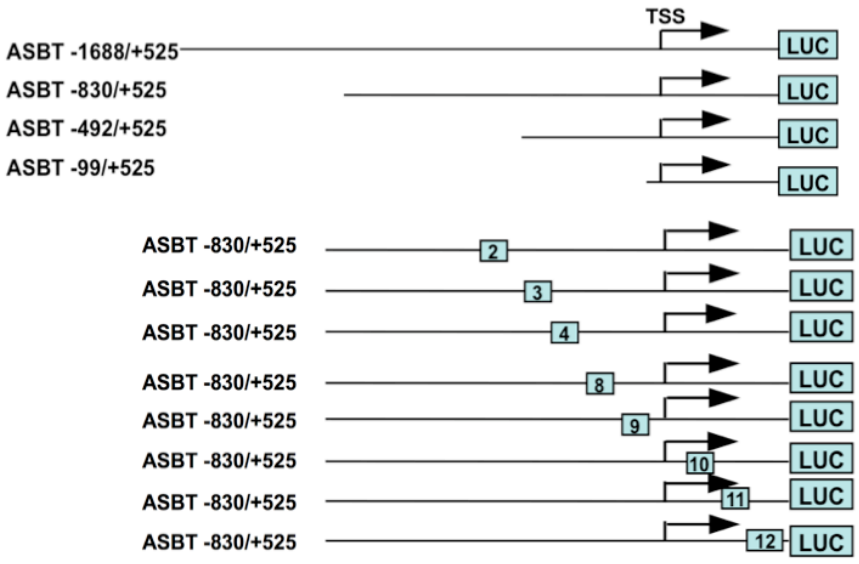



**Fig. 4.5 Region containing the predicted CDX binding sites mediates *ASBT* promoter activation.** Two hundred nanograms of the CDX1 or CDX2 expression plasmids were transfected into cells, together with 400 ng of the *ASBT* (-830/+525) or *ASBT* (-99/+525) promoter luciferase construct. Reporter activities were measured 36 h later. *A*, T84 cells; *B*, OE19 cells; *C*, Het1A cells. **P*<0.05; ****P*<0.001.

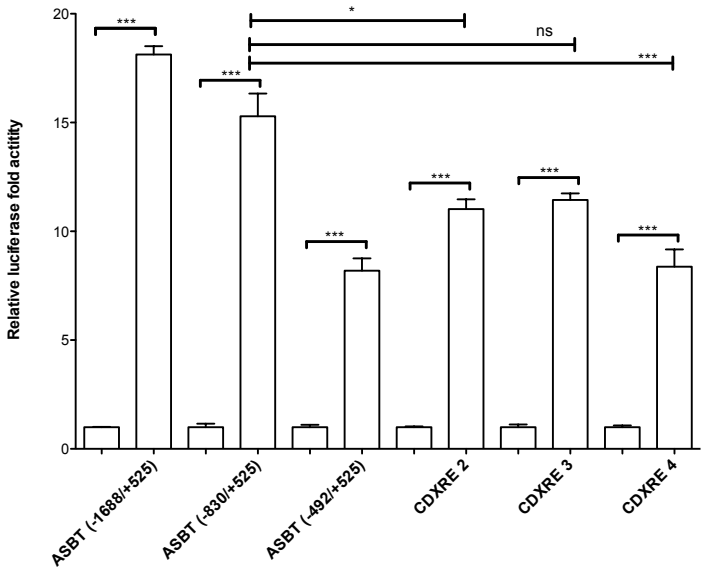
### *4.1.6 Identification of the CDX binding sites that mediate ASBT promoter activation*

To study further which of the predicted *CDXRE*s mediated CDX-dependent activation in the proximal promoter region, I created eight *ASBT* promoter constructs in which critical point mutations were introduced to the individual *CDXRE*s by site-directed mutagenesis using the *ASBT* (-830/+525) promoter construct as a PCR template (Fig 4.6*A*). As neither CDX1 nor CDX2 were able to bind to the *CDXRE1, CDXRE5,* or *CDXRE6 in vitro* (see below), mutation constructs were not created for these binding sites. In transient transfections in Caco-2 cells, the construct *ASBT* (-830/+525), which contained all the predicted *CDXRE*s, was potently activated by both CDX1 and CDX2, and the most significant loss was observed when mutations were introduced to *CDXRE4* in the proximal promoter (Fig. 4.6*B*). In the case of *CDXRE*s in the UTR, mutation of the binding site *CDXRE12* showed the most pronounced reduction of CDX activation (Fig. 4.6*C*). However, no single *CDXRE* mutation could entirely abolish the activation in response to CDX, suggesting that more than one *CDXRE* mediate *ASBT* promoter activation.

**A**



**B**


Relative luciferase fold actitity
20
15
10
5
0
***
*
ns
***
***
***
***
***
***
ASBT (-1688/+525)
ASBT (-830/+525)
ASBT (-492/+525)
CDXRE 2
CDXRE 3
CDXRE 4

**C**

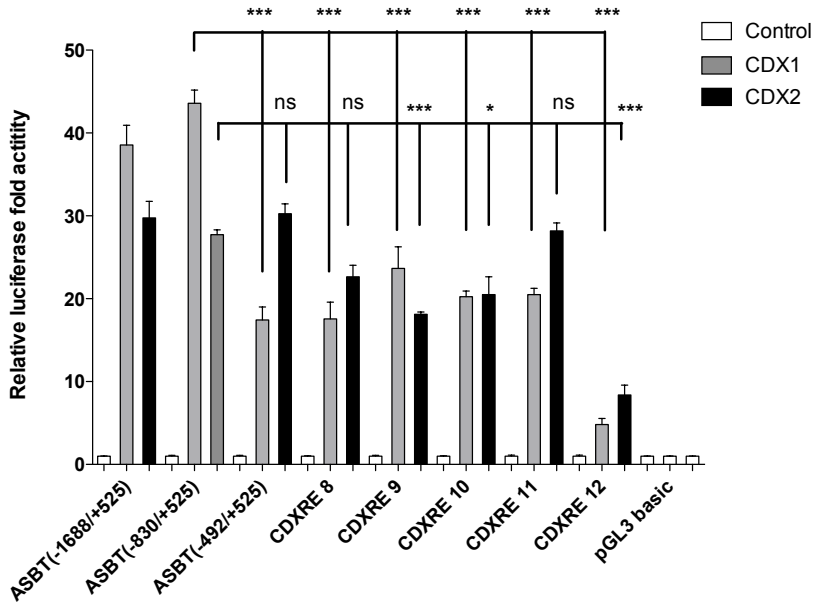

Relative luciferase fold actitity
50
40
30
20
10
0
Control
CDX1
CDX2
***
***
***
***
***
***
ns
ns
***
*
ns
***
ASBT(-1688/+525)
ASBT(-830/+525)
ASBT(-492/+525)
CDXRE 8
CDXRE 9
CDXRE 10
CDXRE 11
CDXRE 12
pGL3 basic

**Fig. 4.6 Identification of functional CDX binding sites that mediate human *ASBT* promoter activation.** *A*, Schematic view of *ASBT* deletion constructs; the box with a number indicates that point mutations were introduced at the specific binding site by site-directed mutagenesis. The short vertical line with arrow indicates transcription start site (TSS). *B*, Caco-2 cells were cotransfected with a reporter-linked *ASBT* promoter deletion construct -1688/+525, -830/+525, or -492/+525, or the -830/+515 construct containing mutations in either *CDXRE2*, *CDXRE3*, or *CDXRE4* within the proximal promoter region. With each construct, either a *CMV* promoter-driven expression plasmid for CDX1, or an empty pcDNA3.1(+) vector (control) was cotransfected. *C*, Caco-2 cells were cotransfected with reporter-linked *ASBT* promoter construct -1688/+525, -830/+525, -492/+525, *CDXRE 8*, *CDXRE 9*, or *CDXRE10–12* in the 5' UTR region, as well as the promoterless reporter vector pGL3basic. With each construct, either a *CMV* promoter-driven expression plasmid for CDX1 and CDX2, or an empty pcDNA3.1(+) vector (control) was cotransfected. Relative luciferase activities obtained for pcDNA3.1(+)-transfected cells were set to 1, and the fold activities in other test conditions are shown relative to these. **P*<0.05; ****P*<0.001; ns, not significant.

### *4.1.7 Synergistic activation of the ASBT proximal promoter by CDX1 and HNF1α*

In a known evolutionarily conserved mechanism, CDXs interact with the transcription factors HNF-1α and GATA4 in regulation of several intestine-specific genes. The following experiments investigated, whether CDX1 functionally interacts with the HNF-1α and/or GATA4 factors in the context of *ASBT* promoter. As expected, CDX1 or HNF-1α alone transactivated the promoter. When CDXs and HNF-1α were simultaneously overexpressed in cells, this led to ~100-fold activation, which greatly exceeded that induced by CDXs or HNF-1α alone or additively. It is interesting to note that transfection of cells with the GATA4 expression plasmid significantly reduced the synergism between CDX1 and HNF-1α (Fig 4.7).

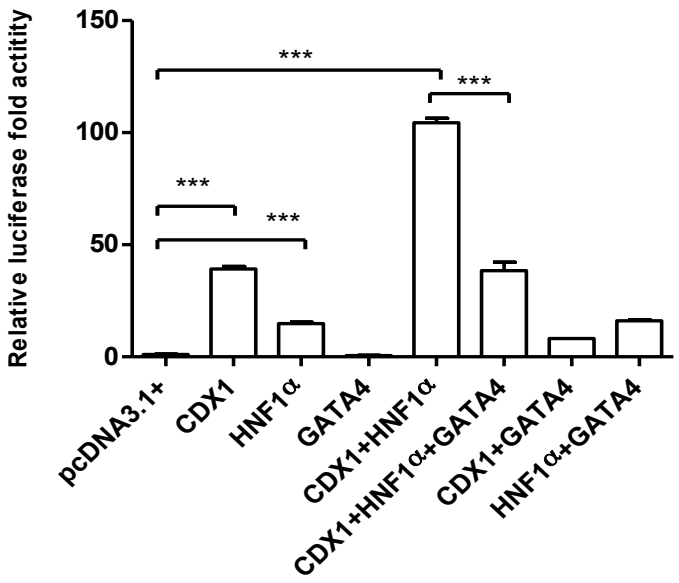



**Fig. 4.7 Cooperative transcriptional regulation by CDX1, HNF-1α, and GATA4 on the *ASBT* promoter.** Two hundred nanograms of CDX1, HNF-1α, and/or GATA4 expression plasmids were transfected into cells. In relevant cases, the total amount of CMV promoter-containing DNA was adjusted by adding pcDNA3.1(+) construct, together with 400 ng *ASBT* (-1688/+525) promoter luciferase construct. Reporter activities were measured 36 h later. ****P*<0.001.

### *4.1.8 Antibody specificity tests in EMSAs*

To confirm the specificity of CDX1 and CDX2 antibodies used in EMSA, I tested the cross-reactivity of the antibodies: CDX1 antibody with *in vitro* translated CDX2 proteins, and CDX2 antibody with *in vitro* translated CDX1 proteins (Fig. 4.8). Loss of DNA-binding was observed when CDX1 antibody was added to the CDX1 protein mixture (lane 2). By addition of either of the two CDX2 antibodies did not interfere with CDX1 protein binding to DNA (lanes 3 and 4). In a reciprocal manner, when adding CDX2 antibody to CDX2 protein binding reactions, CDX2 proteins were supershifted thus forming larger and slower migrating protein complexes (lanes 6 and 7), whereas adding CDX1 antibody did not alter or reduce CDX2 protein-probe complex formation (lane 8).

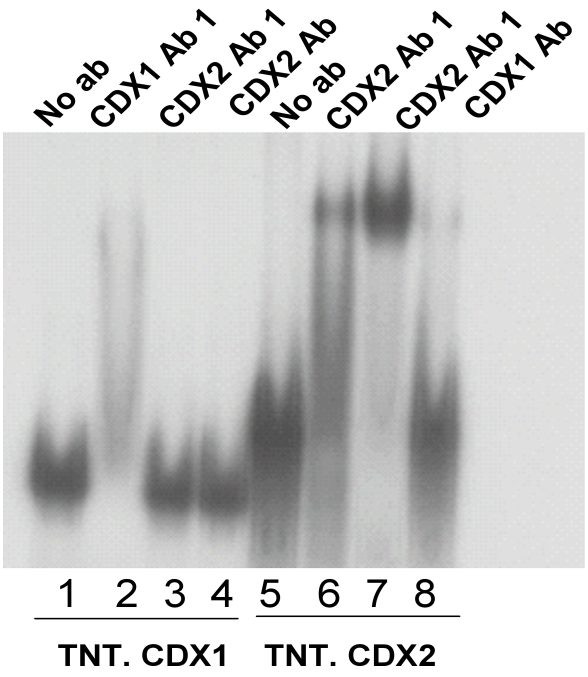



**Fig. 4.8 CDX1 and CDX2 antibodies specifically recognize the respective proteins.** EMSAs were carried out using *in vitro* translated CDX1 and CDX2 proteins. CDX consensus binding site from the human *desmocollin 2* gene was used as a radiolabelled probe. The specificity of the complexes was confirmed by incubation with an anti-CDX1 antibody or two different anti-CDX2 antibodies.

### *4.1.9 CDX1 and CDX2 bind to six of the nine predicted CDXREs on the ASBT promoter in vitro*

To study the potential *in vitro* interaction between CDX1 and CDX2 with their predicted binding motifs within the *ASBT* promoter, I performed EMSAs using nuclear extracts derived from LS174T cells, in which both CDX1 and CDX2 are endogenously expressed, using wild-type and mutant predicted *CDXRE*s as radiolabeled probes. Two DNA-protein complexes were formed on the *CDXRE*s 2, 3, 4, 7, 8, and 9, which were abolished by addition of either CDX1 (faster complex) or CDX2 (slower complex) antibodies (Fig. 4.9*A*). Addition of unrelated antibodies raised against the nuclear receptor transcription factors PPARα or RXRα did not affect CDX–DNA complex formation. When mutations designed to interfere with CDX DNA-binding were introduced into the oligonucleotides and used as radioactively labelled probes, the formation of the two CDX-DNA complexes was abolished or strongly reduced. The other putative *CDXRE*s within the proximal *ASBT* promoter (*CDXRE*s 1, 5, and 6) predicted *in silico* failed to form specific complexes with CDXs in EMSAs (data not shown). To further confirm the specificity of CDX1 and CDX2 binding to the *CDXRE*s within the *ASBT* promoter, I performed EMSA competition experiments (Fig. 4.9*B*), using the CDX consensus binding site from the human *desmocollin 2* gene as a radiolabelled probe, and increasing molar excesses of unlabelled competitor oligonucleotides harbouring the *ASBT* promoter *CDXRE*s. The binding of recombinant *in vitro* translated CDXs to their consensus binding site was competed off in a dose-dependent manner, upon addition of competitor oligonucleotides containing each wild-type *CDXRE* of the *ASBT* promoter. Mutated CDX elements were incapable of, or significantly less efficient in, competing for CDX binding. These data indicate that CDXs are able to bind specifically to *CDXRE*s in the proximal *ASBT* promoter sequence *in vitro*.

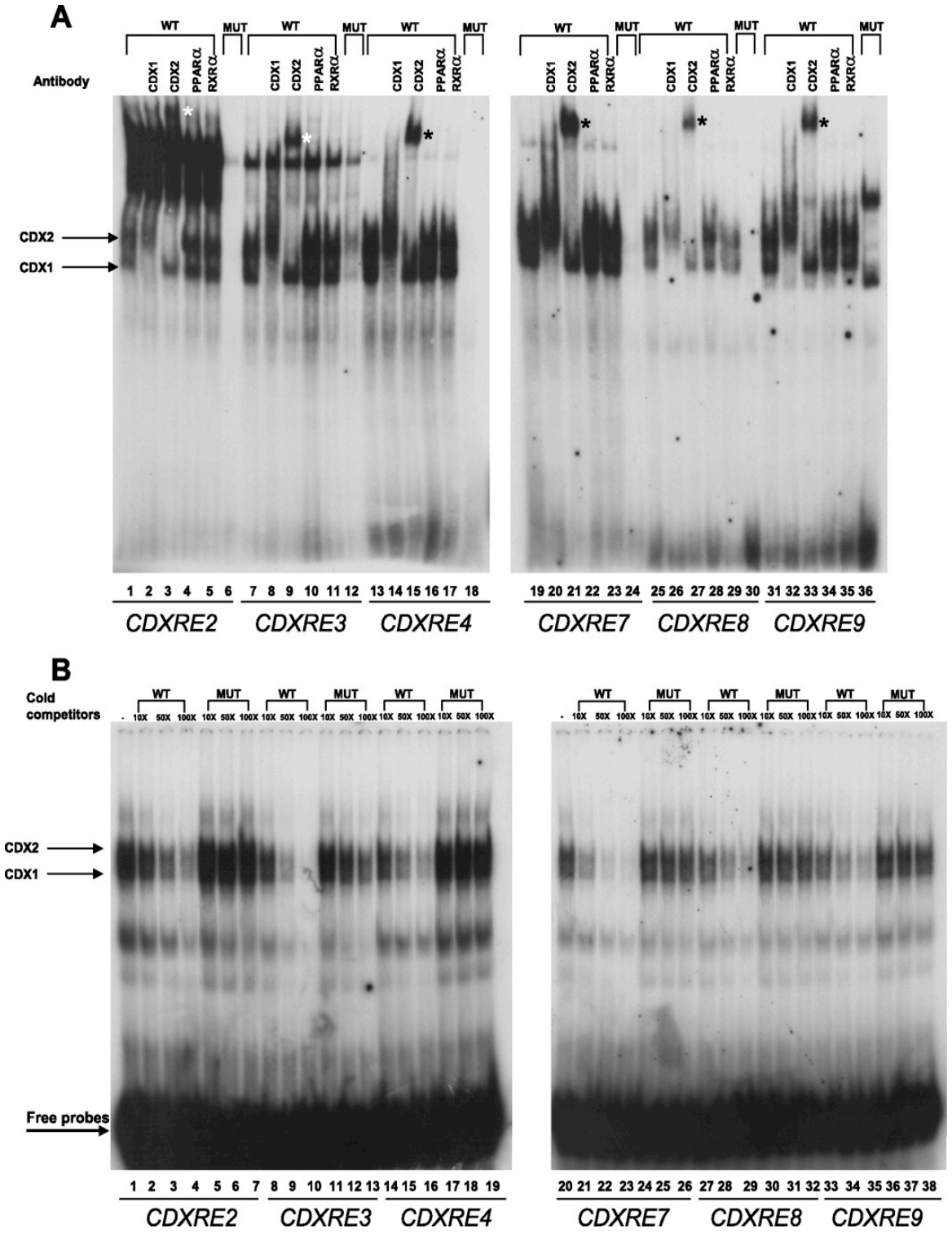

A
Antibody
WT
MUT
CDX1
CDX2
PPARα
RXRα
CDX2
CDX1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36
CDXRE2
CDXRE3
CDXRE4
CDXRE7
CDXRE8
CDXRE9
B
Cold competitors
10X 50X 100X
CDX2
CDX1
Free probes
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
CDXRE2
CDXRE3
CDXRE4
CDXRE7
CDXRE8
CDXRE9

**Fig. 4.9 CDX1 and CDX2 bound to six predicted *CDXRE*s *in vitro*.** EMSAs were carried out using nuclear extracts from LS174T cells. Oligonucleotides containing the six predicted wild-type or mutant *CDXRE*s within the proximal *ASBT* promoter were radioactively labelled for use as probes. CDX1 and CDX2 complexes are indicated by black arrowheads (lanes 1, 7, 13, 19, 25, and 31). The specificity of the complexes was confirmed by incubation with an anti-CDX1 (lanes 2, 8, 14, 20, 26, and 32) or anti-CDX2 (lanes 3, 9, 15, 21, 27, and 33) antibody. To confirm the specificity of the protein-DNA complexes, as well as the anti-CDX antibodies used in supershifts, anti-PPARα (lanes 4, 10, 16, 22, 28, and 34) and anti-RXRα (5, 11, 17, 23, 29, and 35) antibodies were tested in parallel. The asterisks indicate the location of CDX2-probe complexes supershifted with antibodies. The anti-CDX1 antibody abolished the CDX1-probe complexes, rather than produced a defined supershift. The interaction between the radiolabelled probes and CDXs was abolished or strongly reduced by mutations to crucial bases within CDX binding sites (lanes 6, 12, 18, 24, 30, and 36). The two EMSA image panels were derived from the same experiment and from film exposure times of equal length. *B*, Competition EMSAs were carried out using *in vitro* translated CDX1 and CDX2 proteins, both simultaneously added to the binding reactions. Unlabelled oligonucleotides containing the *CDXRE*s from the human *ASBT* promoter were added in 10-, 50-, or 100-fold molar excess. The oligonucleotide containing the consensus *CDXRE* derived from the human *desmocollin 2* promoter was used as the radioactively labelled probe. The two EMSA image panels were derived from the same experiment and from film exposure times of equal length.

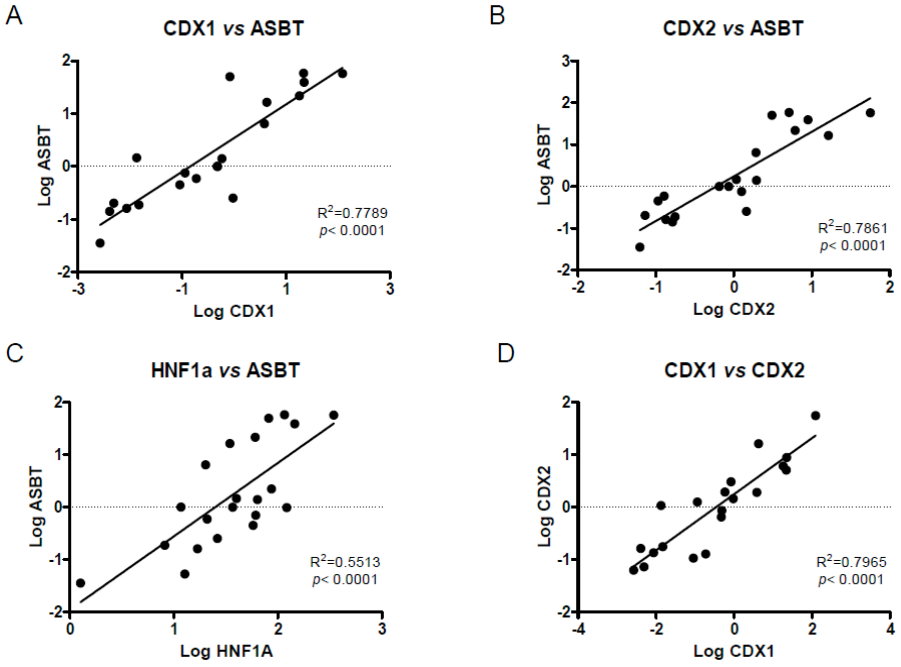
### *4.1.10 CDX1 and CDX2 interact with the proximal ASBT promoter region within living cells*

To confirm the interaction between CDX1 and CDX2 with the *ASBT* promoter in living cells, ChIP assays were performed using nuclear proteins extracted from T84 cells in which CDX1, CDX2 and ASBT are all endogenously expressed. As shown in Fig. 4.10 (upper panel), two different CDX1 and two CDX2 antibodies were efficient in precipitating the promoter region *ASBT* (-736/-154), whereas the nonspecific mouse IgG antibodies failed to precipitate the *ASBT* promoter region. As an additional specificity control, I amplified a 221 bp region from the first intron of the *ASBT* gene using the same ChIP samples as templates. None of the CDX antibodies was able to precipitate this intronic region of the *ASBT* gene (Fig. 4.10, lower panel).


**Fig. 4.10 CDX1 and CDX2 interact with the proximal, CDX-responsive, *ASBT* promoter region within living cells.** ChIP assays were performed in T84 cells. Two different CDX1- and CDX2-specific antibodies, but not the negative control antibody (mouse IgG), were efficient in precipitating the promoter region *ASBT* (-736/-154) (upper panel). None of the antibodies could precipitate the intronic region located approximately eight kbps downstream of the transcriptional start site of the *ASBT* gene (lower panel).

### *4.1.11 Correlation analysis of CDX, ASBT, and HNF-1α expression levels in human BE and ileal tissue*

To study whether CDXs or the known transactivator of the *ASBT* promoter, HNF-1α [150], are chief factors in maintaining the expression of ASBT mRNA in human BE tissue, I measured the expression of all four genes in RNA isolated from esophageal biopsies derived from BE patients. Tissue was obtained from 35 patients, and 20 expressed mRNAs of all genes of interest. ASBT mRNA levels were significantly correlated with those of CDX1 (Fig. 4.11*A*), CDX2 (Fig. 4.11*B*), and HNF-1α (Fig. 4.11*C*). I observed a strong and significant correlation between the mRNA expression levels of CDX1 and CDX2 (Fig. 4.11*D*). In addition to the BE tissue, I also investigated the potential correlation between CDX, HNF-1α and ASBT expression levels in ileal tissue derived from 48 healthy subjects. ASBT mRNA levels were not significantly correlated with those of either CDX1 (Fig. 4.11*E*) or CDX2 (Fig. 4.11*F*), although there was a significant correlation between ASBT and HNF-1α mRNA expression (Fig. 4.11*G*). Similar to the BE tissue, there was a significant correlation between mRNA expression levels of CDX1 and CDX2 in the healthy ileum (Fig. 4.11*H*).



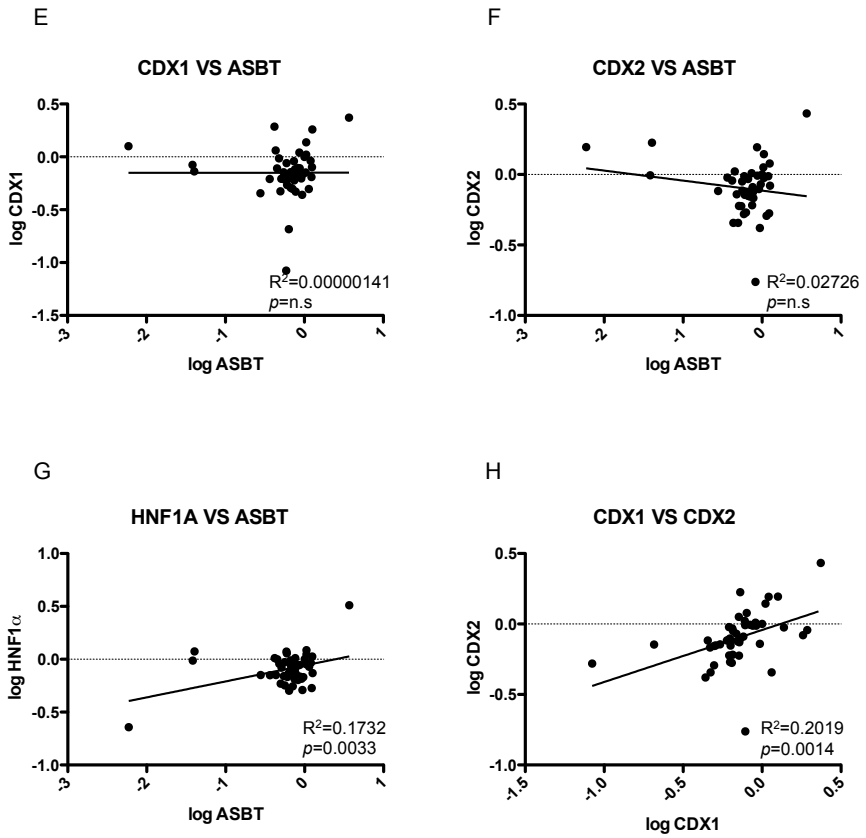
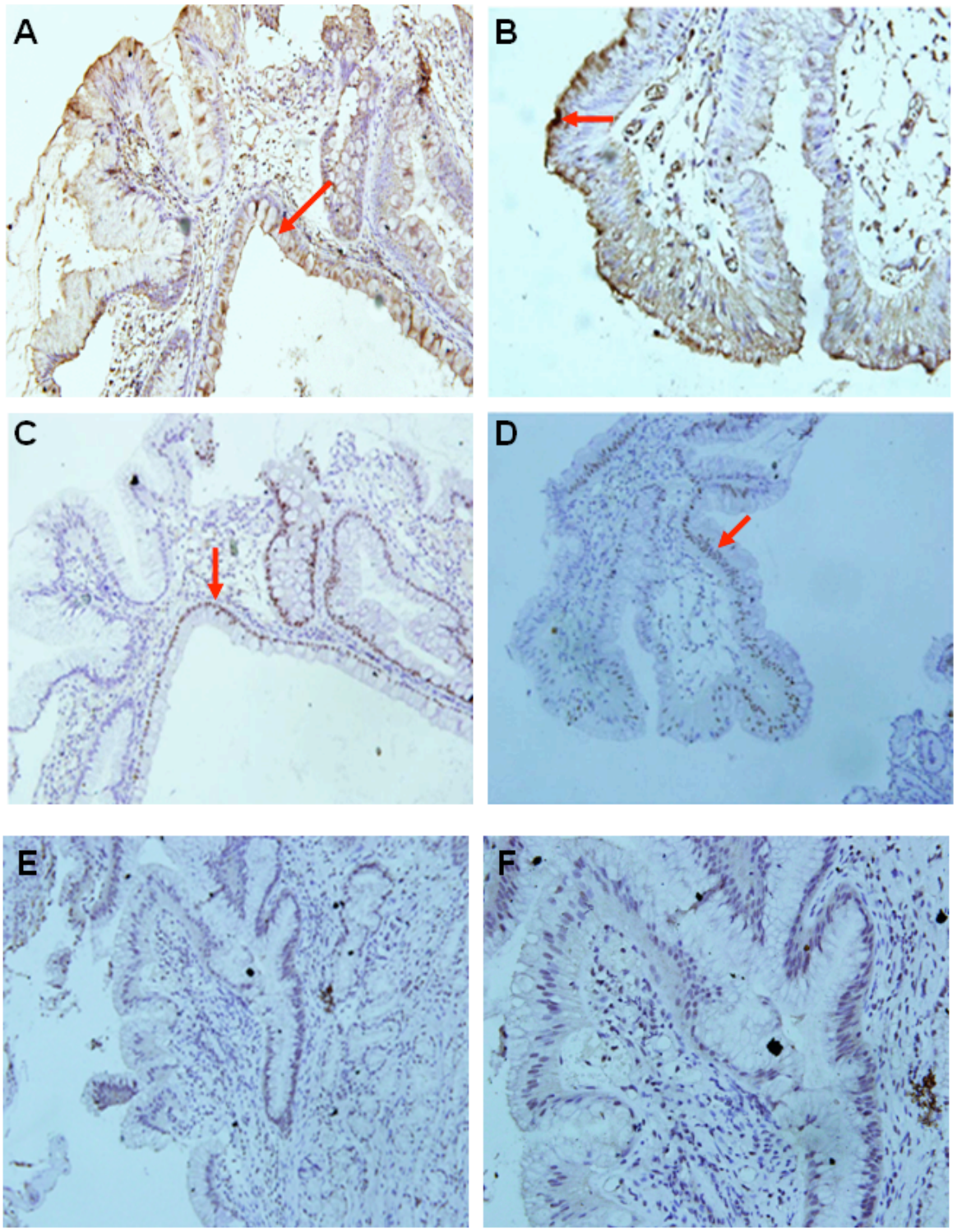



**Fig. 4.11 Correlation of CDX1 (*A*), CDX2 (*B*) and HNF-1α (*C*) mRNA expression levels with those of ASBT in BE biopsies from 20 subjects, using linear regression analysis.** *D*, Correlation analysis of CDX1 and CDX2 mRNA levels with each other in BE tissue. $R^2$ values and *P* values: *A*, $R^2$=0.7789, *P*<0.0001; *B*, $R^2$=0.7861, *P*<0.0001; *C*, $R^2$=0.5513, *P*<0.0001; *D*, $R^2$=0.7965, *P*<0.0001. β-Actin was used as a housekeeping gene for normalization in esophageal material. Correlation of CDX1 (*E*), CDX2 (*F*) and HNF-1α (*G*) mRNA expression levels with those of ASBT mRNA in ileal biopsies from 48 healthy subjects, using linear regression analysis. *H*, Correlation of CDX1 and CDX2 mRNA levels in ileal tissue. $R^2$ values and *P* values: *E*, $R^2$ = 0.00000141, *P*=0.9936; *F*, $R^2$=0.02726, *P*=0.2621; *G*, $R^2$=0.1732, *P*=0.0033; *H*, $R^2$=0.2019, *P*=0.0014. Villin was used as the housekeeping gene for normalization in ileal material. Logarithmically transformed values of CDX1, CDX2, HNF-1α, and ASBT mRNA levels were used.

### *4.1.12 Immunostaining for CDXs and ASBT proteins in formalin-fixed paraffin-embedded (FFPE) blocks from BE tissues*

I attempted to localize CDXs and ASBT proteins in tissue sections from FFPE blocks derived from esophagus of BE patients. All tissue samples contained distinguishable BE features, namely goblet cells and columnar cells. Although no staining for ASBT was detected in the columnar cells, some was observed in goblet cells (Fig. 4.12*A*,*B*). Therefore, it seems possible that the human ASBT antibody available to us generated nonspecific staining, and optimized staining will be needed to validate this. I also observed specific nuclear staining for CDX2 as expected in tissue sections with columnar and goblet cells (Fig. 4.12*C*,*D*). However, none of the tissue sections could be stained with the CDX1 antibody (Fig. 4.12*E*,*F*).




**20X** **40X**

**Fig. 4.12 Protein localization of human ASBT, CDX1, and CDX2 in tissue section from FFPE blocks of BE patients.** *A*, *B,* Staining for the ASBT protein. *C, D,* Nuclear staining for the CDX2 protein. *E, F*, Staining for the CDX1 protein. Antibodies used: ASBT antibody (Paul Dawson Laboratory, Wake Forest, USA), CDX2 antibody (Maixin MAB-0216, Xiamen, China), and CDX1 antibody (AP6130a; Abgent, San Diego, CA, USA).

## 4.2 Regulation of the gene encoding the intestinal bile acid transporter OSTα/β by the caudal-type homeobox proteins CDX1 and CDX2.

### *4.2.1 In silico promoter analysis of the OSTα/β gene promoters*

As shown previously, CDXs can regulate the *ASBT* promoter. Therefore it was of interest to investigate whether CDXs play a role in promoter regulation of the genes encoding intestinal BA export proteins, namely OSTα/β. First, I analyzed the proximal 927 bp of the human *OSTα* promoter (NCBI Reference Sequence: *NT_029928*) and human 1453 bp *OSTβ* promoter (NCBI Reference Sequence: *NW_925884*), to identify putative *CDXRE*s. The consensus core motif for CDX DNA binding is 5′-*TTTA*$^{T}/_{C}$-3′ or 5′-$^{G}/_{A}$*TAAA*-3′ [86]. We identified two putative *CDXRE*s within the proximal *OSTα* (-927/+1) and six in the *OSTβ* (-1453/+1) promoter regions by visual inspection (Fig. 4.13 *A*,*B*).

**A**

**-927** ctctccacaccccacagctttctgtctgacctggtttcttagcctctctggacctcagcc

**-866** tccttatctgtaaaatgggaataaggatactacttcccttgtaacgcggtg**gtaaa**gttt
*CDXRE2*

**-805** aaataagctaaggaacacactgcatgtcctgcgggccctggcacaccagacatgctgaca

**-744** gacgctggctgctttcatcagctatgcaaccaaattctaaggggcccttcgtatgtgtca

**-683** ggctgcgtgccaggaacctagaggtaccaccgtaccacagtacaagatgctgtgtctggc

**-622** ttcgaagagatttcagtctatgtggagagagagaaaagggaagctatcagcca**ataaa**at
*CDXRE1*

**-561** gtttgcaagcctgtaattcagcaggttttcaagacctgtcaaatgcccagcaatctcac

**-500** tctgcaactctgcctcttaaatacagcatcttccggcccagcgtgggggtggctaagccc

**-439** atcatccagatgtgatgaactggggtctgggaaacccacaccggcctcatctggcttttc

**-378** cagcctgtcctcccttccacacatcaggctgacagcaccaaacacggccgaggcctcagc

**-317** cctgagtctccccgctgcagagtgcccaagcctgagttccagcaggttcttcaggggccg

**-256** ctgaccagggaacactgcggtgaggagcgaggacttcgtccccacccggcccagggcag

**-195** gggcttcgtggtgagcccaccccaccccagaaggctcctggaagggcagggcagactggc

**-134** agcaaaatcctataggatgggccgggccagaggcaaggggtggccaaaggcacctgtcca

**-73** agaggtgggagggaccacacctgggggcagggacgagtcaggcacagggaactccgccag

**-12** cctagaggatca**a**attcctccggggtgaactctgagatagaaagttggcccgggaagctc
**TSS**

**B**

**-1453** cgatcctccctgtacacttttttcttggtgccctatggcatgt**tttat**aattgatctgt
*CDXRE6*

**-1392** tcaaaacacttctcttgttttattttagttacg**tttat**ctccattcctactcccttcctc
*CDXRE5*

**-1331** ctcctccccaagacacagcttaatgtgtttaatgcgtgtccttttaaaaaaatgtgttat

**-1270** tgggtcaggcgcggtggctcactcctgtaatcccagtactttgggaggccaaggcgggtg

**-1209** gatcacctgaggtcaagagttcgagaccagcctggccaacatggtgaaaccccatctcta

**-1148** ctaaaaatacaaaaattagtggggcggtagtgacatgcgcctgtaattccagctacttag

**-1087** gaggctgagacaggagaatcgcttgaacctgggaggcggaggttgcagtgagcagagatc

**-1026** gtgccactgcactccagcctgggcgacagagtgagactccgtctcaaaaaaaaaaaaaaa

**-965** aaagcgaaataacttaacaattttaatattggcctgaataatatatgttgtcaaaattaa

**-904** t**tttac**ctatttctttttgttttt**gtaaa**gtatggatgctagaaaattttatattaagtag
*CDXRE4* *CDXRE3*

**-843** taataatgcagttagtacaagaagacgtcaaaatgtctgactgttttgagattgactgag

**-782** gttcagtttattattaatagttgggatggaaataggcctacacgaagtaaggga**tttat**c
*CDXRE2*

**-721** cagagagcatggattcaagttcttcccttgcaaataacagaaaccaattctgctaacttg

**-660** tccaaaaaaaaaaaaaaaaagaaaaaaaaagctctcggggtcggggcaggggtgccctct

**-599** cagcatcaggagccggcagctgcctttccctccggcacttttccctttctcctctctgt

**-538** gtcgtaggcgtgccaatccccaggagagaggcgtgataggcccagcctttgacatgtggg

**-477** gaaacacaggcctgtgattggctaccgcatgacacccaagggacaagttgctatggcaac

**-416** gcgtgggcgcgctgtgggctcgtagaagccaggtgcggacgctgaacaggcaggcgaaga

**-355** cccgagatgtcctttgcaagggtcccagaactcgccagtggctcagtcctcctcactccc

**-294** agaacagtgctcctccacacatgcgatggcttttaa**ataaa**tatttgagtcactcccactt
*CDXRE1*

**-233** ccagtctctgcccctccaggctccacgtgctcctgggttcgttcgggtcgtttttgtccc

**-172** ataactttgggccagaggagatctggatttgggcacagagggagatcaaactcgggtggg

**-111** atatccagtcatgctgtctgctttgtttccaggggtcttcacggcttctctgcccagggg
**TSS**

**-50** ccagaaccgaggaggccaggagggctgctggggctaaggggtctaaggac**c**tcgttgcac

**Fig 4.13 *In silico* analysis of the *OSTα/β* promoter regions.** *A*, The sequence of the proximal *OSTα* promoter region (-927/+48). The transcription start site is indicated by "TSS". Two predicted *CDXRE*s were found, and they are shown in bold. The positions of the predicted *CDXRE*s were as follows: *CDXRE1* (-568/-564) and *CDXRE2* (-814/-810). *B*, Sequence of the proximal *OSTβ* promoter region (-1435/+9). The transcription start site is indicated by "TSS". The consensus motifs found in the six predicted *CDXRE*s are shown in bold. The positions of the predicted *CDXRE*s were as follows: *CDXRE1* (-258/-254), *CDXRE2* (-727/-723), *CDXRE3* (-981/-977), *CDXRE4* (-899/-903), *CDXRE5* (-1357/-1353), and *CDXRE6* (-1408/-1404).

### *4.2.2 CDX1 and CDX2 modulate endogenous OSTα/β at the mRNA level*

Specifically targeted knocking down of endogenous CDX1 and CDX2 expression levels in T84 cells, in which both CDXs and OSTα/β are endogenously expressed, resulted in significantly reduced endogenous OSTα mRNA expression (Fig. 4.14*A*). Double knockdown of CDX1 and CDX2 did not significantly reduce the OSTα expression level, whereas knocking down CDX1 and CDX2 both individually and in combination reduced the OSTβ mRNA levels (Fig. 4.14*B*). This suggested that CDXs regulate OSTα/β expression at the transcriptional level.

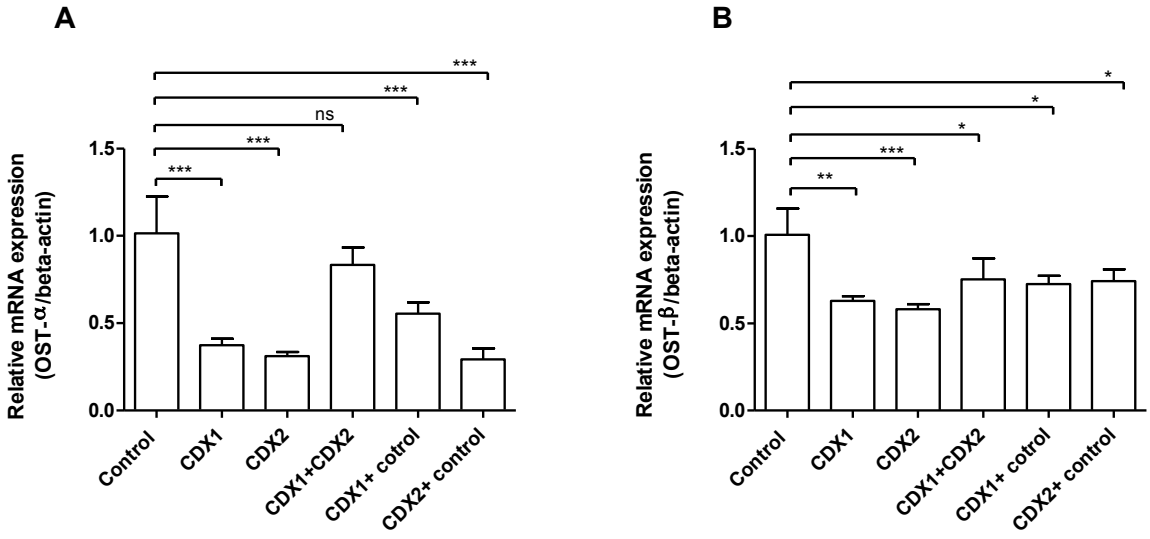



**Fig. 4.14 Modulation of endogenous OSTα/β mRNA expression levels by CDX1 and CDX2.** *A*, OSTα mRNA expression was reduced upon knockdown of endogenous CDX1 and CDX2 expression in T84 cells in comparison to control siRNA-transfected cells. *B*, OSTβ mRNA expression was reduced upon knockdown of endogenous CDX1 and CDX2 expression in T84 cells in comparison to control siRNA-transfected cells. Cells were transfected with control non-targeting siRNA, siCDX1, or siCDX2 at a final concentration of 50 nM, twice at a 24-h interval, and harvested after 48 h. OSTα, OSTβ, CDX1, and CDX2 mRNA expression was normalized to that obtained for β-actin. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ns, not significant.

### *4.2.3 CDX1 and CDX2 regulate OSTα/β promoters in a cell line-dependent manner*

In Caco-2 cells, both CDX1 and CDX2 transactivated the *OSTα* promoter, but CDXs had no effect on the *OST-β* promoter (Fig. 4.15*A*). However, CDX2, but not CDX1, transactivated the *OSTα/β* promoters in LS174T cells (Fig. 4.15*B*). Similarly to Caco-2 cells, in DLD1 cells both CDX1 and CDX2 transactivated the *OSTα* promoter, but not the *OSTβ* promoter, despite several putative binding sites being found on the latter promoter region (Fig. 4.15*C*).

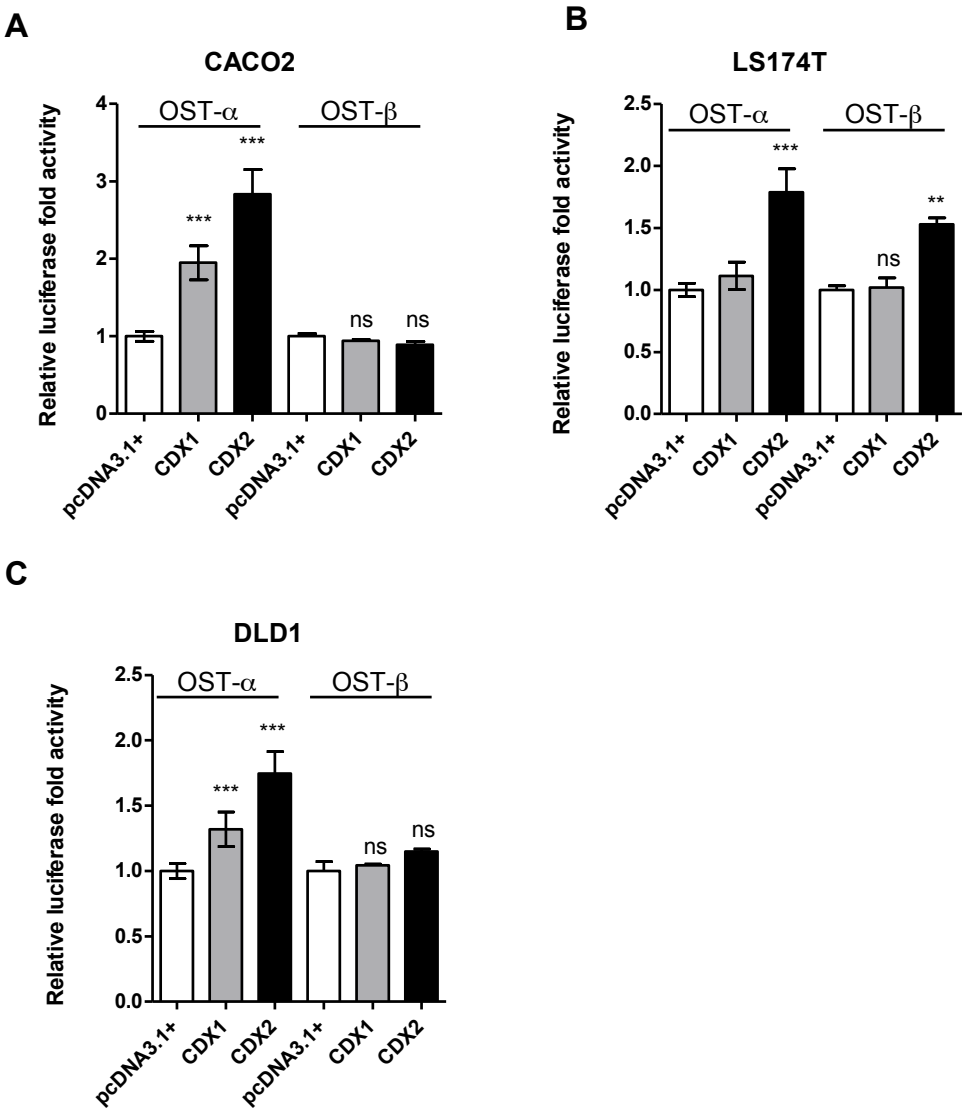


**Fig. 4.15 CDX1 and CDX2 transactivate the human *OSTα* and *OSTβ* promoters in a cell line-dependent manner.** Two hundred nanograms of CDX1 or CDX2 expression plasmids were transfected into Caco-2 (*A*), LS174T (*B*), and DLD1 (*C*) cells, together with 400 ng of *OSTα* (-1457/+161) or *OSTβ* (-4748/+29) promoter luciferase constructs. Reporter activities were measured 36 h later. Transfected *OSTα/β* promoters are indicated on the top of each graph. **, $P<0.01$; ***, $P<0.001$; ns, not significant.

### *4.2.4 Correlation analysis of CDX and OSTα/β expression levels in human BE tissue*

To study whether CDXs are chief factors in maintaining the expression of *OSTα/β* mRNA in human BE tissue, I measured their expression in RNA isolated from esophageal biopsies derived from BE patients. Tissue was obtained from 45 patients who expressed mRNAs of all four genes of interest. OSTα/β mRNA levels significantly correlated with those of CDX1 (Fig. 4.16*A,B*) and CDX2 expression levels only correlate significantly with OSTβ, not OSTα, although even in the latter case there is a clear tendency (Fig. 4.16*C,D*).

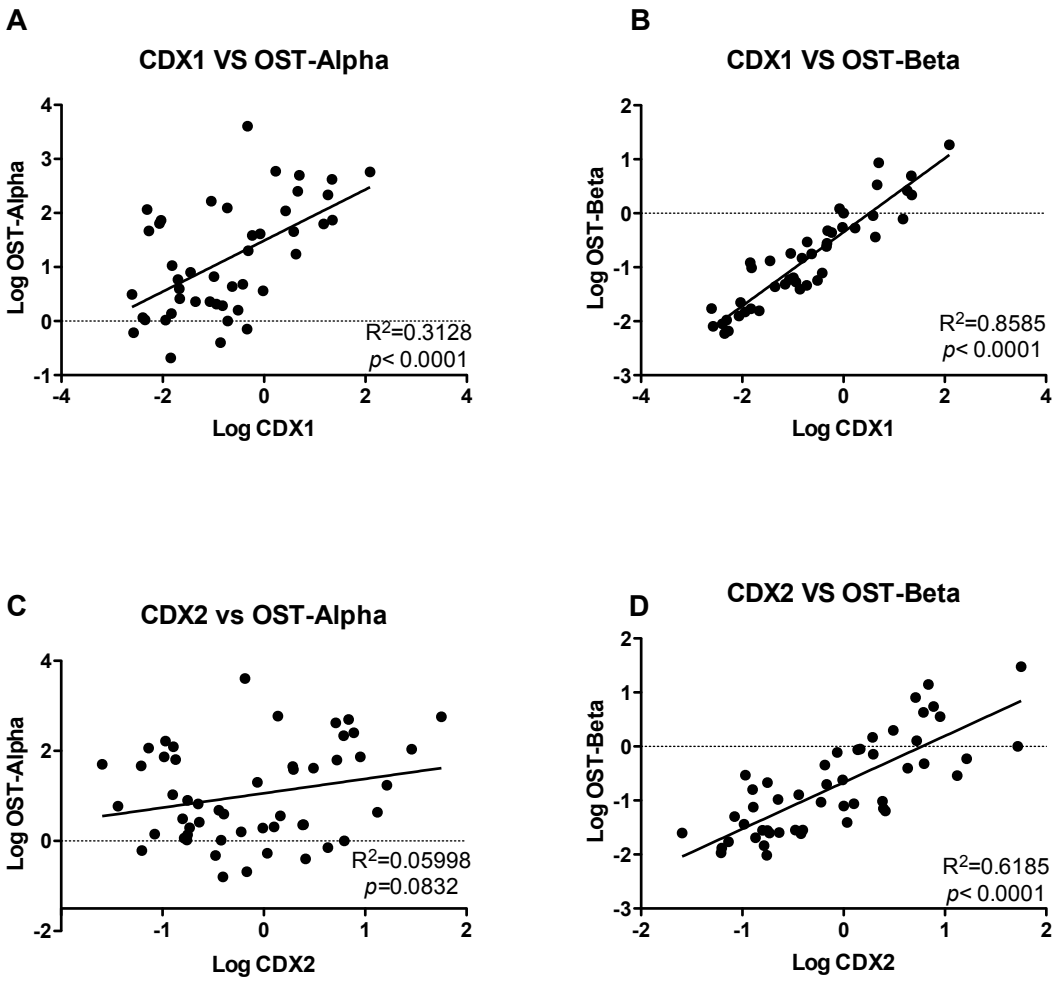



**Fig. 4.16 Correlation of CDX1 *versus* OSTα (*A*), CDX1 *versus* OSTβ (*B*), CDX2 *versus* OSTα (*C*), and CDX2 *versus* OSTβ (*D*) mRNA expression levels in BE biopsies from 45 patients, using linear regression analysis.** $R^2$ values and *P* values: *A*, $R^2$=0.3128, *P*<0.0001; *B*, $R^2$=0.8585, *P*<0.0001; *C*, $R^2$=0.05998, *P*<0. 0.0832; *D*, $R^2$=0.6185, *P*<0.0001. β-actin was used as a housekeeping gene for normalization in esophageal material. Logarithmically transformed values of CDX1, CDX2, OSTα, and OSTβ mRNA levels were used.

## 4.3 FW SCREENING

### *4.3.1 Quality control of long-distance PCR amplification*

Construction of the cDNA library to be employed in the FW screening for novel interactors of CDXs was performed by a PCR-based method. To evaluate the size distribution, yield and quality of the library, a sample of 5 μl from the library was run in an agarose gel.

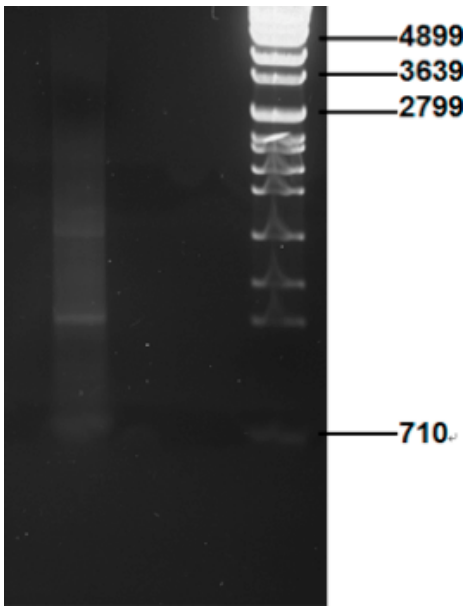



**Figure 4.17 Long-distance PCR to assess the quality of cDNA library construction.** Upon completion of long-distance PCR, a 5 μl sample of the PCR product was analyzed alongside DNA Molecular Weight Marker VII (Roche), on a 1.1% agarose/ethidium bromide gel. Double-stranded cDNA should appear as a 0.1-4 kbp smear on the gel.

### *4.3.2 Determination of the number of pfu of the unamplified cDNA library*

As shown in the calculation, characterization of the library revealed it to be of high quality. The current unamplified library contained ~$4.5\times10^6$ independent clones, whereas a typical unamplified cDNA library has ~$10^6$ independent clones.

| Ratio | pfu/ml |
|---|---|
| 1: 2 | $120x16=1920x2x0^3=3.84x10^6$ pfu/ml |
| 1:5 | $164x6=984x5x10^3=4.92x10^6$ pfu/ ml |
| 1:10 | $76x4=304x10x10^3=3.04x10^6$ pfu/ ml |
| 1:20 | $77+50+66+70=263x20x10^3= 5.26x10^6$ pfu/ ml |
| 1:50 | $27x4=108x\ 50x10^3=5.4x10^6$ pfu/ ml |
| | Average = $4.5\times10^6$ pfu/ ml |

### *4.3.3 Determination of the number of pfu of the amplified cDNA library*

As shown in the calculation, after amplification of the unamplified library, it was expanded to ~35-fold of the original library.

| Ratio | pfu/ml |
|---|---|
| Plate 1: | $74x10^4x10^3/ 5= 1.48x\ 10^8$ pfu/mL |
| Plate 2: | $150x10^4x10^3/ 10= 1.5x10^8$ pfu/mL |
| Plate 3: | $97x\ 4\ quarters=388x10^4x10^3 =1.94\ x10^8$ pfu/mL |
| Plate 4: | Control no plaque |
| | Average = $1.6\times10^8$ pfu/mL |

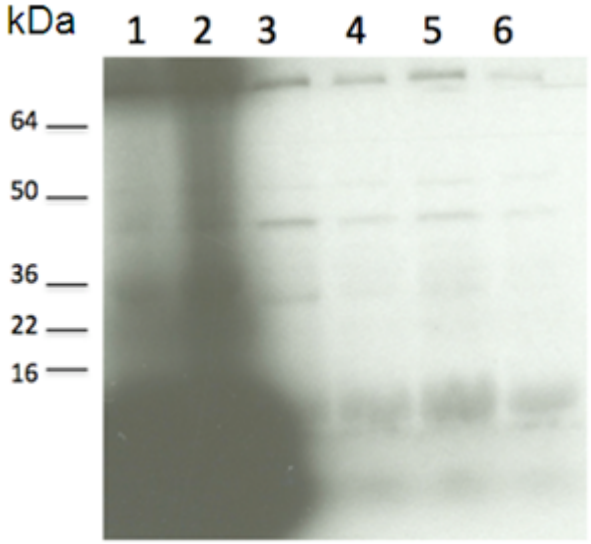
### *4.3.4 Determination of the percentage of recombinant clones*

Three blue plaques out of total of 920 were found on the plate; therefore, the percentage of recombination, and insert-containing clones, was (920-3)/920 = 99.6%.

### *4.3.5 In vitro translation of pTNT-CDX1 and pTNT-CDX2*

*In vitro* translation of CDX1 was first carried out using the T7 promoter-containing original pcDNA3.1(+)-CDX1 vectors, no specific recombinant proteins of adequate quality or expected molecular weight could generated (Fig. 4.18A). The coding sequences of CDX1 and CDX2 were then subcloned into the pTNT vector bearing features that enhanced protein translation, namely the 5′-UTR leader sequence of the β-globin gene and a synthetic poly (A) tail, that enhance the transcriptional and translational efficiency. The proteins were translated using rabbit reticulocyte lysate and wheat germ extracts, that both contains necessary component of the protein translation machinery. In terms of translation efficiency and quality of the CDX1 and CDX2 protein product, no major differences were observed between the two systems. Reticulocyte lysate system was selected for the actual experiment in FW screening (Fig. 4.18B).

**A**



**B**

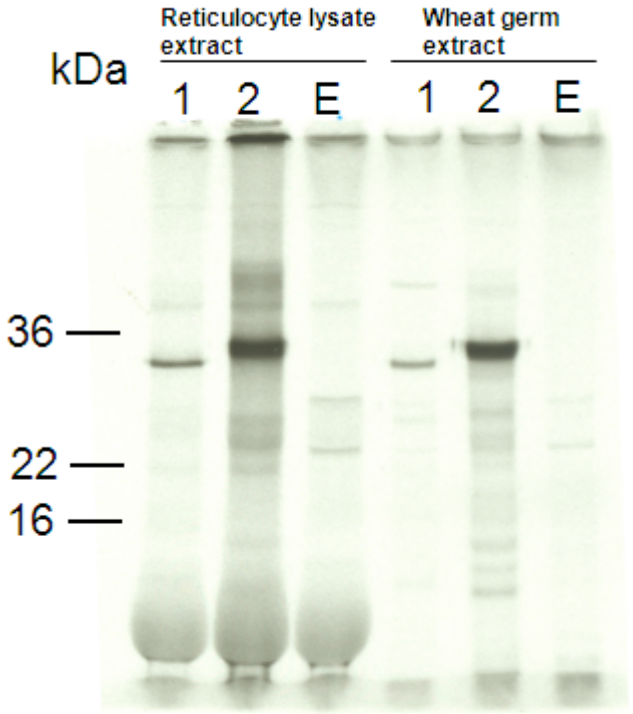



**Fig. 4.18 Comparison of *in vitro* translation of CDX1 and CDX2 proteins using TNT coupled reticulocyte lysate systems and TNT coupled wheat germ extract system.** *A*, Lane 1&2: The pcDNA3.1(+)-CDX1 plasmid used as the template before purification by the Microcon centrifugal filtration units; Lane 3&4: CDX1 proteins after purification by the Microcon units with a molecular weight cut-off of 3 kDa; Lane 5&6: CDX1 proteins after purification by the Microcon units with a molecular weight cut-off of 10 kDa. *B*, Lanes 1: The pTNT-CDX1 plasmid used as the template for the *in vitro* translation reactions. The CDX1 protein was expected to migrate at 28 kDa. Lanes 2: The pTNT-CDX2 plasmid used as the template for the *in vitro* translation reactions. The CDX2 protein was expected at to migrate at 33 kDa; Lanes E: The pTNT vector without an insert used as the template for the *in vitro* translation reactions.

### *4.3.6 Purification of in vitro translated CDX1 and CDX2 proteins*

The translated proteins were purified with a Microcon centrifugal filtration units to remove any molecules smaller than 3 or 10 kDa by centrifugation at speed of 14,000 rpm. As can be seen from Fig 4.19, the excess $^{35}$S-Met and $^{35}$S-Cys were efficiently removed upon the purification step. Ten kDa Microcon units were selected for the actual purification process in FW screening.

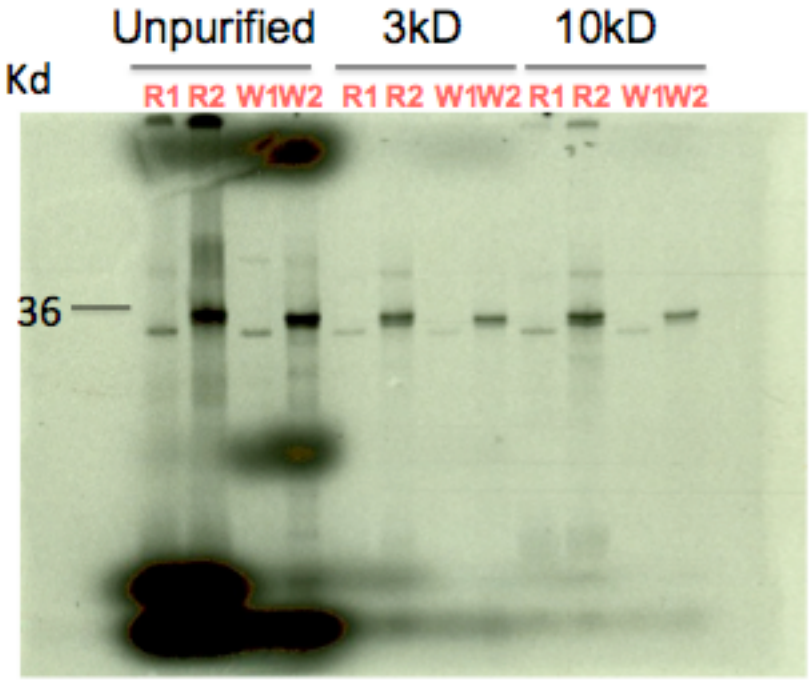



**Fig. 4.19 Purification of *in vitro* translated CDXs proteins with Microcon centrifugal filter units.** Comparison of unpurified samples, samples purified using 3 kDa pore Microcon units, and samples purified using 10 kDa pore Microcon units. R1 = rabbit reticulocyte lysates synthesizing CDX1; R2 = Rabbit reticulocyte lysates synthesizing CDX2; W1 = Wheat germ extracts synthesizing CDX1; W2 = Wheat germ extracts synthesizing CDX2.

### *4.3.7 Far western screening*

We next performed the primary FW screening of $1.7\times10^6$ clones for each CDX protein. After the secondary screening, for the positive clones, the λTriplEx recombinant phages were converted to plasmids via excision and circularization. The plasmids were released by Cre recombinase-mediated recombination at *loxP* sites. Excision of the insert-containing plasmid occurred automatically, when recombinant phages were transduced into an *E. coli* host BM25.8 expressing the Cre recombinase. Conversion was performed on individual positive plaques selected from the secondary screening plate. Excised plasmids were then propagated stably in a recombinase-deficient *E. coli* strain. Screening of a small intestine and colon cDNA library ($1.7\times10^6$ clones) using radioactive protein probes corresponding to the complete coding region of the human transcription factors CDX1 and CDX2 resulted in identification of 15 and 6 positive plaques in the primary screen, respectively. These were selected and subjected to two consecutive additional rounds of plaque purification. Following conversion of 42 plaque-purified clones from λTriplEx to pTriplEx, the insert sizes were determined by restriction analysis of the plasmid DNAs. The sequences of the inserts were identified by DNA sequencing, using oligonucleotide primers flanking the insert cloning site in pTriplEx. cDNAs listed in Table 4.1 encoded possible CDX1 interaction partners, and those in Table 4.2 possible CDX2 interaction partners.

Table 4.1 List of putative CDX1 interaction partners.

| Clone number | Reference sequence | Encoded/predicted protein | Known/proposed protein functions |
|---|---|---|---|
| 15A1 | ref\|NT_011525.7\| | Bromodomain containing protein 1 | Component of the MOZ/MORF complex which has a histone H3 acetyltransferase activity. |
| 15B1 | ref\|NM_001037165.1\| | Forkhead box K1 | Transcriptional regulator that binds to the upstream enhancer region (CCAC box) of myoglobin gene. Has a role in myogenic differentiation and in remodeling processes of adult muscles that occur in response to physiological stimuli |
| 16A1 | ref\|NM_018302.2\| | Chromosome 4 open reading frame 19 | N/A |
| 16B1 | ref\|NM_022463.3\| | nucleoredoxin | Regulate the Wnt/*β*-catenin pathway, which regulates cell fate and early development. |
| 17A1 | ref\|NR_003680.1\| | ribosomal protein L13A pseudogene | Ribosomal protein. |
| 17B1 | ref\|NM_002473.3\| | MYH9 | Myosin, heavy chain 9, non-muscle. |

| | | | |
|---|---|---|---|
| 18A1 | ref\|NM_018464.2\| | CISD1 | CDGSH iron sulphur domain 1. Plays a key role in regulating maximal capacity for electron transport and oxidative phosphorylation. May be involved in Fe-S cluster shuttling and/or in redox reactions. |
| 18B1 | ref\|NM_080748.2\| | C20orf52 | *Homo sapiens* chromosome 20 open reading frame 52. |
| 19A1 | ref\|NM_020729.1\| | ODF2L | *Homo sapiens* outer dense fiber of sperm tails 2-like. |
| 19B1 | ref\|NT_009952.14\|Hs13_10109 | *Homo sapiens* chromosome 13 genomic contig, reference assembly. | N/A |
| 20A1 | ref\|NM_001012.1\| | RPS8 | *Homo sapiens* ribosomal protein S8. |
| 20B1 | No significant similarity found | No significant similarity found. | N/A |

Table 4.2 List of putative CDX2 interaction partners.

| Clone number | Reference sequence | Encoded/predicted protein | Known/proposed protein functions |
|---|---|---|---|
| 1 | ref\|NW_001842393.1\|HsX_WGA1354_36 | *Homo sapiens* chromosome X genomic contig, alternate assembly . | |
| 2 | ref\|NW_001838967.1\|Hs5_WGA360_36 | *Homo sapiens* chromosome 5 genomic contig, alternate assembly. | |
| 3 | ref\|NM_006565.2 | *Homo sapiens* CCCTC-binding factor (zinc finger protein) (CTCF), | 11 Zinc finger protein |
| 4 | No significant similarity found. | | |
| 5 | ref\|NT_022184.14\|Hs2_22340 | *Homo sapiens* chromosome 2 genomic contig, reference assembly. | Mitochondrial trifunctional protein, alpha subunit precursor. |
| 6 | ref\|NW_001838877.2\|Hs3_WGA270_36 | *Homo sapiens* chromosome 3 genomic contig, alternate assembly. | Hypothetical protein. |
| 7 | ref\|NW_001838967.1\|Hs5_WGA360_36 | *Homo sapiens* chromosome 5 genomic contig, alternate assembly. | Heterogeneous nuclear ribonucleoprotein H1 |
| 8 | ref\|NM_000690.2 | *Homo sapiens* aldehyde dehydrogenase 2 family (mitochondrial) (ALDH2), nuclear gene encoding mitochondrial protein, mRNA. | |

| | | | |
|---|---|---|---|
| 9 | ref\|NW_001842393.1\|HsX_WGA1354_36 | *Homo sapiens* chromosome X genomic contig, alternate assembly . | Hypothetical protein. |
| 10 | ref\|NT_005403.16\|Hs2_5560 | *Homo sapiens* chromosome 2 genomic contig, reference assembly. | Glutaminase. |
| 11 | ref\|NW_001842393.1\|HsX_WGA1354_36 | *Homo sapiens* chromosome X genomic contig, alternate assembly. | Hypothetical protein. |
| 12 | ref\|NW_001842393.1\|HsX_WGA1354_36 | *Homo sapiens* chromosome X genomic contig, alternate assembly. | Hypothetical protein. |
| 13 | No significant similarity found. | | |
| 14 | ref\|NW_001842393.1\|HsX_WGA1354_36 | *Homo sapiens* chromosome X genomic contig, alternate assembly. | Hypothetical protein. |
| 15 | ref\|NW_001842393.1\|HsX_WGA1354_36 | *Homo sapiens* chromosome X genomic contig, alternate assembly. | Hypothetical protein |
| 16 | No significant similarity found. | N/A | N/A |
| 17 | No significant similarity found. | N/A | N/A |
| 18 | No significant similarity found. | N/A | N/A |
| 19 | ref\|NM_016426.4\| | GTSE1 | Homo sapiens G-2 and S-phase expressed 1 |

| | | | |
|---|---|---|---|
| 20 | No significant similarity found. | N/A | N/A |
| 21 | ref\|NM_006360.3 | *Homo sapiens* eukaryotic translation initiation factor 3, subunit. | Eukaryotic translation initiation factor 3, subunit M. |
| 22 | ref\|NM_005756.2 | GPR64, transcript variant 4 | *Homo sapiens* G protein-coupled receptor 64 |
| 23 | ref\|NW_001838967.1\|Hs5_WGA360_36 | H1 | Heterogeneous nuclear ribonucleoprotein |
| 24 | ref\|NT_008470.18\|Hs9_8627 | *Homo sapiens* chromosome 9 genomic contig, reference assembly | |
| 25 | ref\|NT_008470.18\|Hs9_8627 | mitogen-activated protein kinase associated protein 1 | |
| 26 | No significant similarity found. | N/A | N/A |
| 27 | No significant similarity found. | N/A | N/A |
| 28 | ref\|NM_001042446.1\| | CAST | *Homo sapiens* calpastatin |
| 29 | ref\|NW_001838967.1\|Hs5_WGA360_36 | H1 | Heterogeneous nuclear ribonucleoprotein |
| 30 | ref\|NM_005756.2\| | GPR64 | *Homo sapiens* G protein-coupled receptor 64 |

# 5 DISCUSSION

## 5.1 Regulation of the gene encoding ASBT by CDX1 and CDX2

Intestinal BA transporters are membrane proteins that are important in regulating the influx and efflux of BA. They are essential components of the enterohepatic circulation of BAs and of cholesterol homeostasis. It is therefore of physiological relevance to study the molecular regulatory mechanisms of the expression of these membrane transporters.

I demonstrated in human intestine- and esophagus-derived cell lines that the human *ASBT* promoter is a direct target for transcriptional activation by the transcription factors CDX1 and CDX2. Knockdown of endogenous CDX1 and CDX2 with siRNAs resulted in reduced endogenous ASBT mRNA expression level (Fig. 4.2). Consistent with this, exogenous expression of CDXs resulted in increased *ASBT* promoter activity in luciferase reporter assays (Fig. 4.3*A*-*C*). The CDX-mediated activation was conserved in the mouse and rat *Asbt* promoters (Fig. 4.3*D*,*E*), and consistent with this, preliminary *in silico* analysis revealed several putative *CDXRE*s in both rodent *Asbt* promoters (data not shown), even though their overall homology with the human *ASBT* promoter was low. The level of activation of the rodent *Asbt* promoters appeared lower than for the human *ASBT* promoter. However, one limitation of the experimental setup was that human CDX expression constructs were used, and these may have had different coactivator requirements from their rodent counterparts. It will be interesting to study whether the intestinal Asbt expression and consequently BA absorption is reduced in Cdx mutant mice, although it should be noted that complete Cdx1/Cdx2-null mice cannot be analyzed for this purpose, because they are not viable until birth. It is interesting to note that the CDX-mediated activation was conserved in the mouse and rat *Asbt* promoters. Consistent with this, preliminary *in silico* analysis revealed several putative *CDXRE*s in both rodent *Asbt* promoters (data not shown).

I identified several putative *CDXRE*s within the proximal human *ASBT* promoter in *in silico* analysis (Fig. 4.4), and deletion of the promoter region harbouring these motifs significantly reduced transactivation by CDXs (Fig. 4.5). Of the seven promoter constructs containing individually mutated *CDXRE*s all showed reduction in CDX-mediated activation compared to the control vector, although disruption of any single element did not lead to complete abolition of *ASBT* promoter activation (Fig. 4.6). It is of advantage for a given factor or particular gene promoters to have more than one response element: 1) this may allow step-wise activation depending on the occupancy of the response elements on the gene promoter, 2) this may compensate for loss of a binding site caused by DNA damage, or to prevent the effects caused by a polymorphism occurring within a single response element. Six of these predicted *CDXRE*s could bind both recombinant and endogenous nuclear CDX proteins *in vitro* (Fig. 4.8 and 4.9). The region containing these six novel CDX binding sites also interacted with both CDX1 and CDX2 within living cells, as shown in ChIP assays (Fig. 4.10). In healthy human ileal tissue, there was no correlation between mRNA expression of the CDX transcription factors and ASBT (Fig. 4.11*E*,*F*), indicating that CDXs may not be crucial for maintaining the baseline ASBT expression level in normal human ileum, but may rather be important in *ASBT* promoter regulation in different tissues in response to specific signalling pathways, or in certain pathological conditions, such as BE. A correlation has been previously reported between ASBT and CDX2 in ileal tissue of patients suffering from chronic diarrhea [205]. In contrast to CDXs, the mRNA levels of HNF-1α were significantly correlated with ASBT mRNA expression levels in the ileum (Fig. 4.11*G*), confirming that HNF-1α contributes to the baseline expression of ASBT. This is consistent with reports that HNF-1α-null mice have severely reduced Asbt expression, and that ileal HNF-1α protein levels correlate with ASBT mRNA levels in humans [206]. Ileal CDX1 and CDX2 expression levels were also significantly correlated with each other (Fig. 4.11*H*), supporting the prior suggestion that they mutually regulate of one another expression [106].

CDX1 and CDX2 play important roles not only in early gastrointestinal development, but also in gastrointestinal diseases in adult humans. Their ectopic overexpression in esophageal and stomach metaplasia suggests that they play a role in the pathogenesis of these diseases [207]. Alternatively, CDX expression could be the consequence rather than the cause of metaplasia.

Given the elevated and ectopic expression of both CDXs and ASBT in BE, we studied whether CDXs acted as regulators of the *ASBT* promoter. In human esophageal and intestine-derived cell lines, the human *ASBT* promoter was a direct target for transcriptional activation by the transcription factors CDX1 and CDX2. My results add the intestinal BA transporter, *ASBT*, to the list of genes regulated by CDXs. Other intestinal transporters regulated by CDXs include peptide transporter 1 (PEPT1) [103] and sodium-coupled monocarboxylate transporter (SMCT)-1 [104]. Furthermore, the present research adds another component to the already known complexity of transcriptional regulation of *ASBT* gene expression.

Cooperative synergism between CDX1 and HNF-1α in the activation of the *ASBT* promoter is shown in Fig 4.7. In this context, it is interesting to note that it has been previously proposed that HNF-1α and CDX2 cooperate with each other in the regulation of the intestinal genes *sucrase isomaltase* [96] and *calbindin-D9k* [208]. It is currently not known whether the CDXs interact with the previously identified regulators of the *ASBT* promoter, such as GR [44], HNF-1α, and PPARα [45].

The correlation between the mRNA expression levels of CDXs and ASBT was confirmed in BE biopsy material. The role of BA in the molecular pathogenesis of esophageal metaplasia remains somewhat unclear, although BAs has been shown to augment directly expression of CDX1 [106] and CDX2 [162]. It may thus be that secondary to gastrointestinal reflux, the BAs that enter the esophagus lead to elevated ASBT expression via induction of their transactivator CDXs. Excess level of BAs are toxic and have been shown to be carcinogenic to the gastrointestinal tract [209], and liver [210-212], and BAs are the major component of the duodenal juice, which can cause severe esophageal mucosal damage [145]. Dvorak *et al.* have demonstrated that ASBT, IBABP and MRP3 are elevated in BE and decreased in EAC, at both the mRNA and protein levels. It has been proposed that BA transporter expression in gastrointestinal metaplasia of the esophagus serves as a protective mechanism by the host to remove excess BAs secondary to chronic biliary reflux.

As in healthy human ileal tissue, CDX expression did not correlate with ASBT expression, this regulatory mechanism may be more relevant in BE than in healthy ileum. Gene

expression levels are not maintained by a single transcription factor. As mentioned in the introduction, human ASBT gene expression is known to be transcriptionally regulated by multiple factors. Correlation has been previously reported between ASBT and CDX2 in ileal tissue of patients suffering from chronic diarrhea [205].

## 5.2 Regulation of the gene encoding OSTα/β by CDX1 and CDX2

The mRNA and protein levels of OSTα/β are closely correlated, suggesting that tight transcriptional co-regulation of the two genes is essential for functional protein complex production [85]. It is therefore of physiological relevance to understand the transcriptional activators or repressors that modulate OSTα/β expression. BAs induce *OSTα/β* promoters via acting as ligands for FXR [80]. Two functional FXR response elements were found on the *OSTα* promoter and one on the *OSTβ* promoter [18]. I propose OST*α/β* are other novel target genes for CDX1 and CDX2.

In the second section of the presented work, *in silico* promoter analyses provided support for the hypothesis that CDXs transcriptionally regulate the *OST* genes. Two *CDXRE*s were identified in the *OSTα* proximal promoter region and six in the *OSTβ* proximal promoter region (Fig 4.13A&B). it was shown that, by knocking down endogenous CDX1 and CDX2 in T84 cells, the endogenous levels of OSTα and OSTβ were notably decreased in comparison to control siRNA (Fig. 4.14). This suggests that the *OSTα/β* genes may be transcriptional targets of CDXs. It was also demonstrated that CDX1 and CDX2 transactivated the *OSTα*, but not *OSTβ*, promoter in Caco-2 and DLD1 cells (Fig. 4.15*A,C*). In the LS174T cell line, CDX2 but not CDX1 transactivated the *OSTα*/*β* promoters (Fig. 4.15*B*). Overexpressing CDXs, despite the abundance of putative *CDXRE*s located within the *OSTβ* promoter, did not elevate the OSTβ expression levels. The apparent contradiction in promoter activation could be explained as there might have been missing cofactors in the transcriptional machinery complex within specific cell lines.

CDX1/2 mRNA levels correlate with OST*α/β* level in BE tissue biopsies (Fig 4.16). Taken together, it is interesting to note that both *ASBT* and *OSTα/β* genes thus appear coordinately regulated by the intestinal transcription factors CDX1 and CDX2. I have described here for

the first time the expression of BA efflux transporters OSTα/β in the BE tissue. I speculate that in the microenvironment of BE, ASBT react first to the exposure of BAs, whereas OSTα/β on the opposite of the membrane help to remove excess intracellular toxic BAs, thereby reducing cellular stress. An adaptive response to elevation of OST has been previously described in cholestatic liver disease [213].

Further confirmations are needed before any firm conclusion can be drawn. EMSAs using nuclear extracts from intestinal cells, together with appropriate antibody supershifts, and the putative CDXREs as EMSA probes, as well as ChIP experiments, are required to confirm the *in vitro* and *in vivo* interactions between CDX factors and the *OST* promoter regions.

## 5.3 Far western screening

Identification of protein partners is an important way to understand protein function. In the work described above, a high-quality cDNA library was constructed from commercially available human small intestine and colon mRNA that represented a broad spectrum of proteins in these tissues. The construction involved a SMART technique, which enhanced the full-length cDNA, preserving the 5′ end of the sequence. The cDNA library was subsequently propagated in the lambda phage and the radiolabelled *in vitro* translated protein products were used as probes for FW screening. After primary and secondary screening procedures, I identified potentially positive phage clones, which were isolated from plaques and subsequently sequenced and analyzed with the BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Several different genes were identified, including GPCRs, myosin, ribosomal protein 8, and nucleoredoxin. I found two particularly promising CDX interaction partners that are discussed in more detail below.

The protein “Bromodomain-containing 1” (BRD1) has a highly conserved bromodomain that contains 220 amino acids. A bromodomain consists of four α-helices, and is present in nearly all nuclear histone acetyltransferases (HATs), which are involved in the chromatin structure regulation and gene expression [214]. Bromodomains recognize acetate lysine-containing peptides, components of the SWI/SNF chromatin remodeling complex. Bromodomain-containing ptoteins are epigenetic remodelling factors. CDX2 has been previously shown to

interact directly with the BRM-type SWI/SNF chromatin remodelling complex, and these two proteins cooperatively regulate villin expression in intestinal cells [215].

CTCF is a CCCTC motif-binding transcription factor that was originally identified as a factor that binds to the CT-rich region of the chicken c-myc gene with 11 highly conserved zinc finger DNA-binding domains. Given different cellular settings, CTCF can act either as a transcriptional activator by binding to a HAT-containing complex or function as a transcription repressor by binding to a histone-deacetylase (HDAC) complex [216]. We propose that CTCF may interact with either CDX1 or CDX2, thus regulating their transcriptional activity.

# 6 CONCLUSIONS AND FUTURE PERSPECTIVES

I conclude that CDX1 and CDX2 can regulate the *ASBT* gene at the transcriptional level. Evidence for this regulation and elucidation of the molecular mechanism behind it was obtained by a number of methods, by siRNA-mediated knockdown, exogenous overexpression, EMSAs, ChIP, and correlation of expression in human tissue biopsies. CDX-mediated promoter activation may lead to aberrant esophageal expression of ASBT and consequently to an increase in epithelial BA uptake activity by the mucosa in BE. Furthermore, my findings may provide an explanation for the correlation between the decrease in both ASBT and CDX expression in high-grade esophageal dysplasia. In future studies, cooperation and interactions between CDXs and the other known transcriptional factors of the ASBT promoter - GATA family, PPARs, VDR, and glucocorticoid receptor - would be of interest.

Based on our current preliminary findings, we conclude that CDX1 and CDX2 may also regulate the *OST*α/β promoters, perhaps with ASBT together in a coordinated manner. This regulation may have an important implication in the microenvironment in BE: Upon exposure to BAs, ASBT reacts to remove the toxic BAs, OSTα/β on the other side of the membrane is upregulated to release the stress of intracellular bile acid load by effluxing them out of the cell. EMSAs and ChIPs will be required to confirm the *in vitro* and *in vivo* binding of the CDX factors to the *OST* promoters in relevant cell lines. Deletion constructs of the *OSTα/β* promoter will be required to map the exact CDX-responsive regions, which mediate the activation. The current *ex vivo* expression correlation results using human biopsy material strongly support the original hypothesis.

There are several limitations to the present study. Despite the progress outlined above, there remain important questions that require further study in relation to a more complete understanding of transcriptional regulatory mechanisms of the *ASBT* and *OST* promoters by CDXs. These include:

1. The lack of protein-based evidence in siRNA knockdown experiments or CDX overexpression experiments with regard to endogenous ASBT expression. This is due to the current lack of good CDX1 or CDX2 antibodies available that are suitable for western blotting.

2. I made many attempts to perform immunohistochemical staining of tissue sections from paraffin-embedded blocks with anti-ASBT, anti-CDX1, and anti-CDX2 antibodies, to show co-localization of CDX1 and CDX2 with ASBT in the same cells. Although there appeared to be specific nuclear staining with one of the anti-CDX2 antibodies tested, there were difficulties with anti-CDX1 and anti-ASBT antibodies. None of the anti-CDX1 antibodies tested resulted in efficient staining. The ASBT antibody produced ambiguous staining, which was at least partly nonspecific. Instead of immunohistochemistry, I collected esophageal tissue biopsies from BE patients and performed a quantitative correlation analysis of ASBT and CDX mRNA expression. There was a significant correlation between CDX and ASBT expression levels in BE tissue. Although the mRNA analysis did not absolutely prove co-localization of CDXs and ASBT, it strongly supported this hypothesis, and the model of CDXs playing an important role in regulating ASBT in BE.

3. I was not able to establish exactly which *CDXRE*s were responsible for the CDX-mediated *ASBT* promoter upregulation. Therefore, it is proposed that there is more than one *CDXRE* involved in *ASBT* promoter activation.

Regarding the protein interaction studies, the candidates to interact with CDXs must be validated in further protein-protein interaction assays, for examples, in immunoprecipitation or YTH, also to map the exact interacting domains. The validated interacting partners must then be functionally tested in promoter-reporter assays by co-transfecting either CDX1 or CDX2 with the co-factor candidate with a gene promoter of interests.

# 7 REFERENCES


1. Norlin M, Wikvall K: **Enzymes in the conversion of cholesterol into bile acids**. *Curr Mol Med* 2007, **7**(2):199-218.
2. Chiang JY: **Regulation of bile acid synthesis**. *Frontiers in bioscience : a journal and virtual library* 1998, **3**:d176-193.
3. Fuchs M: **Bile Acid Regulation of Hepatic Physiology - III. Regulation of bile acid synthesis: past progress and future challenges**. *Am J Physiol-Gastr L* 2003, **284**(4):G551-G557.
4. Khurana S, Raufman JP, Pallone TL: **Bile acids regulate cardiovascular function**. *Clinical and translational science* 2011, **4**(3):210-218.
5. Ballatori N, Christian WV, Lee JY, Dawson PA, Soroka CJ, Boyer JL, Madejczyk MS, Li N: **OST alpha-OST beta: A major basolateral bile acid and steroid transporter in human intestinal, renal, and biliary epithelia**. *Hepatology* 2005, **42**(6):1270-1279.
6. Dawson PA, Hubbert M, Haywood J, Craddock AL, Zerangue N, Christian WV, Ballatori N: **The heteromeric organic solute transporter alpha-beta, ost alpha-ost beta, is an ileal basolateral bile acid transporter**. *J Biol Chem* 2005, **280**(8):6960-6968.
7. Hagenbuch B, Dawson P: **The sodium bile salt cotransport family SLC10**. *Pflugers Archiv : European journal of physiology* 2004, **447**(5):566-570.
8. Eloranta JJ, Kullak-Ublick GA: **Coordinate transcriptional regulation of bile acid homeostasis and drug metabolism**. *Arch Biochem Biophys* 2005, **433**(2):397-412.
9. Alrefai WA, Gill RK: **Bile acid transporters: structure, function, regulation and pathophysiological implications**. *Pharm Res* 2007, **24**(10):1803-1823.
10. Dawson PA, Hubbert ML, Rao A: **Getting the mOST from OST: Role of organic solute transporter, OSTalpha-OSTbeta, in bile acid and steroid metabolism**. *Biochim Biophys Acta* 2010, **1801**(9):994-1004.

11. Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, Hull MV, Lustig KD, Mangelsdorf DJ, Shan B: **Identification of a nuclear receptor for bile acids**. *Science* 1999, **284**(5418):1362-1365.
12. Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, Stimmel JB, Willson TM, Zavacki AM, Moore DD *et al*: **Bile acids: natural ligands for an orphan nuclear receptor**. *Science* 1999, **284**(5418):1365-1368.
13. Laffitte BA, Kast HR, Nguyen CM, Zavacki AM, Moore DD, Edwards PA: **Identification of the DNA binding specificity and potential target genes for the farnesoid X-activated receptor**. *J Biol Chem* 2000, **275**(14):10638-10647.
14. Chiang JYL: **Regulation of bile acid synthesis: pathways, nuclear receptors, and mechanisms**. *Journal of Hepatology* 2004, **40**(3):539-551.
15. Fickert P, Zollner G, Fuchsbichler A, Stumptner C, Pojer C, Zenz R, Lammert F, Stieger B, Meier PJ, Zatloukal K *et al*: **Effects of ursodeoxycholic and cholic acid feeding on hepatocellular transporter expression in mouse liver**. *Gastroenterology* 2001, **121**(1):170-183.
16. Eloranta JJ, Kullak-Ublick GA: **The role of FXR in disorders of bile acid homeostasis**. *Physiology (Bethesda)* 2008, **23**:286-295.
17. Boyer JL, Trauner M, Mennone A, Soroka CJ, Cai SY, Moustafa T, Zollner G, Lee JY, Ballatori N: **Upregulation of a basolateral FXR-dependent bile acid efflux transporter OST alpha-OST beta in cholestasis in humans and rodents**. *Am J Physiol-Gastr L* 2006, **290**(6):G1124-G1130.
18. Landrier JF, Eloranta JJ, Vavricka SR, Kullak-Ublick GA: **The nuclear receptor for bile acids, FXR, transactivates human organic solute transporter-alpha and -beta genes**. *Am J Physiol Gastrointest Liver Physiol* 2006, **290**(3):G476-485.
19. Adachi R, Honma Y, Masuno H, Kawana K, Shimomura L, Yamada S, Makishima M: **Selective activation of vitamin D receptor by lithocholic acid acetate, a bile acid derivative**. *Journal of Lipid Research* 2005, **46**(1):46-57.
20. Makishima M, Lu TT, Xie W, Whitfield GK, Domoto H, Evans RM, Haussler MR, Mangelsdorf DJ: **Vitamin D receptor as an intestinal bile acid sensor**. *Science* 2002, **296**(5571):1313-1316.

21. Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterstrom RH *et al*: **An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway**. *Cell* 1998, **92**(1):73-82.

22. Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, Latour A, Liu YP, Klaassen CD, Brown KK, Reinhard J *et al*: **The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity**. *P Natl Acad Sci USA* 2001, **98**(6):3369-3374.

23. Houten SM, Watanabe M, Auwerx J: **Endocrine functions of bile acids**. *Embo J* 2006, **25**(7):1419-1425.

24. Gupta S, Stravitz RT, Dent P, Hylemon PB: **Down-regulation of cholesterol 7alpha-hydroxylase (CYP7A1) gene expression by bile acids in primary rat hepatocytes is mediated by the c-Jun N-terminal kinase pathway**. *The Journal of biological chemistry* 2001, **276**(19):15816-15822.

25. Qiao L, Han SI, Fang YW, Park JS, Gupta S, Gilfor D, Amorino G, Valerie K, Sealy L, Engelhardt JF *et al*: **Bile acid regulation of C/EBP beta, CREB, and c-Jun function, via the extracellular signal-regulated kinase and c-Jun NH2-terminal kinase pathways, modulates the apoptotic response of hepatocytes**. *Mol Cell Biol* 2003, **23**(9):3052-3066.

26. Maruyama T, Miyamoto Y, Nakamura T, Tamai Y, Okada H, Sugiyama E, Nakamura T, Itadani H, Tanaka K: **Identification of membrane-type receptor for bile acids (M-BAR)**. *Biochem Bioph Res Co* 2002, **298**(5):714-719.

27. Kawamata Y, Fujii R, Hosoya M, Harada M, Yoshida H, Miwa M, Fukusumi S, Habata Y, Itoh T, Shintani Y *et al*: **A G protein-coupled receptor responsive to bile acids**. *J Biol Chem* 2003, **278**(11):9435-9440.

28. Cipriani S, Mencarelli A, Chini MG, Distrutti E, Renga B, Bifulco G, Baldelli F, Donini A, Fiorucci S: **The Bile Acid Receptor GPBAR-1 (TGR5) Modulates Integrity of Intestinal Barrier and Immune Response to Experimental Colitis**. *PloS one* 2011, **6**(10):e25637.

29. Wong MH, Rao PN, Pettenati MJ, Dawson PA: **Localization of the ileal sodium-bile acid cotransporter gene (SLC10A2) to human chromosome 13q33**. *Genomics* 1996, **33**(3):538-540.

30. Wong MH, Oelkers P, Craddock AL, Dawson PA: **Expression cloning and characterization of the hamster ileal sodium-dependent bile acid transporter**. *J Biol Chem* 1994, **269**(2):1340-1347.

31. Wong MH, Oelkers P, Dawson PA: **Identification of a mutation in the ileal sodium-dependent bile acid transporter gene that abolishes transport activity**. *J Biol Chem* 1995, **270**(45):27228-27234.

32. Shneider BL, Dawson PA, Christie DM, Hardikar W, Wong MH, Suchy FJ: **Cloning and molecular characterization of the ontogeny of a rat ileal sodium-dependent bile acid transporter**. *The Journal of clinical investigation* 1995, **95**(2):745-754.

33. Kramer W, Stengelin S, Baringhaus KH, Enhsen A, Heuer H, Becker W, Corsiero D, Girbig F, Noll R, Weyland C: **Substrate specificity of the ileal and the hepatic Na(+)/bile acid cotransporters of the rabbit. I. Transport studies with membrane vesicles and cell lines expressing the cloned transporters**. *Journal of lipid research* 1999, **40**(9):1604-1617.

34. Saeki T, Matoba K, Furukawa H, Kirifuji K, Kanamoto R, Iwami K: **Characterization, cDNA cloning, and functional expression of mouse ileal sodium-dependent bile acid transporter**. *J Biochem* 1999, **125**(4):846-851.

35. Hallen S, Branden M, Dawson PA, Sachs G: **Membrane insertion scanning of the human ileal sodium/bile acid co-transporter**. *Biochemistry* 1999, **38**(35):11379-11388.

36. Banerjee A, Swaan PW: **Membrane topology of human ASBT (SLC10A2) determined by dual label epitope insertion scanning mutagenesis. New evidence for seven transmembrane domains**. *Biochemistry* 2006, **45**(3):943-953.

37. Hu NJ, Iwata S, Cameron AD, Drew D: **Crystal structure of a bacterial homologue of the bile acid sodium symporter ASBT**. *Nature* 2011, **478**(7369):408-411.

38. Weinman SA, Carruth MW, Dawson PA: **Bile acid uptake via the human apical sodium-bile acid cotransporter is electrogenic**. *The Journal of biological chemistry* 1998, **273**(52):34691-34695.

39. Dawson PA, Lan T, Rao A: **Bile acid transporters**. *J Lipid Res* 2009, **50**(12):2340-2357.

40. Craddock AL, Love MW, Daniel RW, Kirby LC, Walters HC, Wong MH, Dawson PA: **Expression and transport properties of the human ileal and renal sodium-dependent bile acid transporter**. *Am J Physiol* 1998, **274**(1 Pt 1):G157-169.

41. Dawson PA, Haywood J, Craddock AL, Wilson M, Tietjen M, Kluckman K, Maeda N, Parks JS: **Targeted deletion of the ileal bile acid transporter eliminates enterohepatic cycling of bile acids in mice**. *J Biol Chem* 2003, **278**(36):33920-33927.

42. Chen F, Ma L, Dawson PA, Sinal CJ, Sehayek E, Gonzalez FJ, Breslow J, Ananthanarayanan M, Shneider BL: **Liver receptor homologue-1 mediates species- and cell line-specific bile acid-dependent negative feedback regulation of the apical sodium-dependent bile acid transporter**. *J Biol Chem* 2003, **278**(22):19909-19916.

43. Li H, Chen F, Shang Q, Pan L, Shneider BL, Chiang JY, Forman BM, Ananthanarayanan M, Tint GS, Salen G *et al*: **FXR-activating ligands inhibit rabbit ASBT expression via FXR-SHP-FTF cascade**. *Am J Physiol Gastrointest Liver Physiol* 2005, **288**(1):G60-66.

44. Jung D, Fantin AC, Scheurer U, Fried M, Kullak-Ublick GA: **Human ileal bile acid transporter gene ASBT (SLC10A2) is transactivated by the glucocorticoid receptor**. *Gut* 2004, **53**(1):78-84.

45. Jung D, Fried M, Kullak-Ublick GA: **Human apical sodium-dependent bile salt transporter gene (SLC10A2) is regulated by the peroxisome proliferator-activated receptor alpha**. *J Biol Chem* 2002, **277**(34):30559-30566.

46. Shih DQ, Bussen M, Sehayek E, Ananthanarayanan M, Shneider BL, Suchy FJ, Shefer S, Bollileni JS, Gonzalez FJ, Breslow JL *et al*: **Hepatocyte nuclear factor-1alpha is an essential regulator of bile acid and plasma cholesterol metabolism**. *Nat Genet* 2001, **27**(4):375-382.

47. Chen X, Chen F, Liu S, Glaeser H, Dawson PA, Hofmann AF, Kim RB, Shneider BL, Pang KS: **Transactivation of rat apical sodium-dependent bile acid transporter and increased bile acid transport by 1 alpha,25-dihydroxyvitamin D-3 via the vitamin D receptor**. *Molecular Pharmacology* 2006, **69**(6):1913-1923.

48. Duane WC, Xiong W, Lofgren J: **Transactivation of the human apical sodium-dependent bile acid transporter gene by human serum**. *J Steroid Biochem Mol Biol* 2008, **108**(1-2):137-148.

49. Duane WC, Xiong W, Wolvers J: **Effects of bile acids on expression of the human apical sodium dependent bile acid transporter gene**. *Biochim Biophys Acta* 2007, **1771**(11):1380-1388.

50. Alrefai WA, Sarwar Z, Tyagi S, Saksena S, Dudeja PK, Gill RK: **Cholesterol modulates human intestinal sodium-dependent bile acid transporter**. *Am J Physiol-Gastr L* 2005, **288**(5):G978-G985.

51. Thomas C, Landrier JF, Gaillard D, Grober J, Monnot MC, Athias A, Besnard P: **Cholesterol dependent downregulation of mouse and human apical sodium dependent bile acid transporter (ASBT) gene expression: molecular mechanism and physiological consequences**. *Gut* 2006, **55**(9):1321-1331.

52. Xu GR, Shneider BL, Shefer S, Nguyen LB, Batta AK, Tint GS, Arrese M, Thevananther S, Ma L, Stengelin S *et al*: **Ileal bile acid transport regulates bile acid pool, synthesis, and plasma cholesterol levels differently in cholesterol-fed rats and rabbits**. *Journal of lipid research* 2000, **41**(2):298-304.

53. Neimark E, Chen F, Li XP, Magid MS, Alasio TM, Frankenberg T, Sinha J, Dawson PA, Shneider BL: **c-Fos is a critical mediator of inflammatory-mediated repression of the apical sodium-dependent bile acid transporter**. *Gastroenterology* 2006, **131**(2):554-567.

54. Annaba F, Sarwar Z, Kumar P, Saksena S, Turner JR, Dudeja PK, Gill RK, Alrefai WA: **Modulation of ileal bile acid transporter (ASBT) activity by depletion of plasma membrane cholesterol: association with lipid rafts**. *American journal of physiology Gastrointestinal and liver physiology* 2008, **294**(2):G489-497.

55. Sarwar Z, Annaba F, Dwivedi A, Saksena S, Gill RK, Alrefai WA: **Modulation of ileal apical Na(+)-dependent bile acid transporter ASBT by protein kinase C**. *Am J Physiol-Gastr L* 2009, **297**(3):G532-G538.

56. Beuling E, Kerkhof IM, Nicksa GA, Giuffrida MJ, Haywood J, aan de Kerk DJ, Piaseckyj CM, Pu WT, Buchmiller TL, Dawson PA *et al*: **Conditional Gata4**

**deletion in mice induces bile acid absorption in the proximal small intestine**. *Gut* 2010, **59**(7):888-895.

57. Oelkers P, Kirby LC, Heubi JE, Dawson PA: **Primary bile acid malabsorption caused by mutations in the ileal sodium-dependent bile acid transporter gene (SLC10A2)**. *J Clin Invest* 1997, **99**(8):1880-1887.
58. Wojtal KA, Eloranta JJ, Hruz P, Gutmann H, Drewe J, Staumann A, Beglinger C, Fried M, Kullak-Ublick GA, Vavricka SR: **Changes in mRNA expression levels of solute carrier transporters in inflammatory bowel disease patients**. *Drug Metab Dispos* 2009, **37**(9):1871-1877.
59. Renner O, Harsch S, Schaeffeler E, Schwab M, Klass DM, Kratzer W, Stange EF: **Mutation screening of apical sodium-dependent bile acid transporter (SLC10A2): novel haplotype block including six newly identified variants linked to reduced expression**. *Hum Genet* 2009, **125**(4):381-391.
60. Renner O, Harsch S, Schaeffeler E, Winter S, Schwab M, Krawczyk M, Rosendahl J, Wittenburg H, Lammert F, Stange EF: **A Variant of the SLC10A2 Gene Encoding the Apical Sodium-Dependent Bile Acid Transporter Is a Risk Factor for Gallstone Disease**. *PloS one* 2009, **4**(10).
61. Tonjes A, Wittenburg H, Halbritter J, Renner O, Harsch S, Stange EF, Lammert F, Stumvoll M, Kovacs P: **Effects of SLC10A2 variant rs9514089 on gallstone risk and serum cholesterol levels- meta-analysis of three independent cohorts**. *BMC Med Genet* 2011, **12**:149.
62. Ho RH, Leake BF, Urquhart BL, Gregor JC, Dawson PA, Kim RB: **Functional characterization of genetic variants in the apical sodium-dependent bile acid transporter (ASBT; SLC10A2)**. *Journal of gastroenterology and hepatology* 2011, **26**(12):1740-1748.
63. Lewis MC, Brieaddy LE, Root C: **Effects of 2164U90 on ileal bile acid absorption and serum cholesterol in rats and mice**. *Journal of lipid research* 1995, **36**(5):1098-1105.
64. Chen L, Yao X, Young A, McNulty J, Anderson D, Liu Y, Nystrom C, Croom D, Ross S, Collins J *et al*: **Inhibition of Apical Sodium-Dependent Bile Acid**

**Transporter (Asbt) as a Novel Treatment for Diabetes**. *Am J Physiol Endocrinol Metab* 2011.

65. Simren M, Bajor A, Gillberg PG, Rudling M, Abrahamsson H: **Randomised clinical trial: The ileal bile acid transporter inhibitor A3309 vs. placebo in patients with chronic idiopathic constipation--a double-blind study**. *Aliment Pharmacol Ther* 2011, **34**(1):41-50.
66. Montagnani M, Marangoni A, Roda A, Azzaroli F, Mazzella G, Roda E, Tsivian M, Neri F, Jovani M, Giandinoto M *et al*: **Generation of a novel antibody probe to the apical sodium-dependent bile acid transporter that inhibits ileal bile acid absorption**. *Mol Pharm* 2009, **6**(3):1012-1018.
67. Annaba F, Kumar P, Dudeja AK, Saksena S, Gill RK, Alrefai WA: **Green tea catechin EGCG inhibits ileal apical sodium bile acid transporter ASBT**. *American journal of physiology Gastrointestinal and liver physiology* 2010, **298**(3):G467-473.
68. Izzat NN, Deshazer ME, Loose-Mitchell DS: **New molecular targets for cholesterol-lowering therapy**. *J Pharmacol Exp Ther* 2000, **293**(2):315-320.
69. Charach G, Grosskopf I, Rabinovich A, Shochat M, Weintraub M, Rabinovich P: **The association of bile acid excretion and atherosclerotic coronary artery disease**. *Therap Adv Gastroenterol* 2011, **4**(2):95-101.
70. Higaki J, Hara S, Takasu N, Tonda K, Miyata K, Shike T, Nagata K, Mizui T: **Inhibition of ileal Na+/bile acid cotransporter by S-8921 reduces serum cholesterol and prevents atherosclerosis in rabbits**. *Arterioscler Thromb Vasc Biol* 1998, **18**(8):1304-1311.
71. Bhat BG, Rapp SR, Beaudry JA, Napawan N, Butteiger DN, Hall KA, Null CL, Luo Y, Keller BT: **Inhibition of ileal bile acid transport and reduced atherosclerosis in apoE(-/-) mice by SC-435**. *Journal of Lipid Research* 2003, **44**(9):1614-1621.
72. West KL, Zern TL, Butteiger DN, Keller BT, Fernandez ML: **SC-435, an ileal apical sodium co-dependent bile acid transporter (ASBT) inhibitor lowers plasma cholesterol and reduces atherosclerosis in guinea pigs**. *Atherosclerosis* 2003, **171**(2):201-210.

73. Wang W, Seward DJ, Li LQ, Boyer JL, Ballatori N: **Expression cloning of two genes that together mediate organic solute and steroid transport in the liver of a marine vertebrate**. *P Natl Acad Sci USA* 2001, **98**(16):9431-9436.

74. Seward DJ, Koh AS, Boyer JL, Ballatori N: **Functional complementation between a novel mammalian polygenic transport complex and an evolutionarily ancient organic solute transporter, OSTalpha-OSTbeta**. *J Biol Chem* 2003, **278**(30):27473-27482.

75. Houten SM, Volle DH, Cummins CL, Mangelsdorf DJ, Auwerx J: **In vivo imaging of farnesoid X receptor activity reveals the ileum as the primary bile acid signaling tissue**. *Molecular Endocrinology* 2007, **21**(6):1312-1323.

76. Soroka CJ, Ballatori N, Boyer JL: **Organic solute transporter, OSTalpha-OSTbeta: its role in bile acid transport and cholestasis**. *Semin Liver Dis* 2010, **30**(2):178-185.

77. Ballatori N, Li N, Fang F, Boyer JL, Christian WV, Hammond CL: **OST alpha-OST beta: a key membrane transporter of bile acids and conjugated steroids**. *Front Biosci* 2009, **14**:2829-2844.

78. Li N, Cui ZF, Fang F, Lee JY, Ballatori N: **Heterodimerization, trafficking and membrane topology of the two proteins, Ost alpha and Ost beta, that constitute the organic solute and steroid transporter**. *Biochemical Journal* 2007, **407**:363-372.

79. Rao A, Haywood J, Craddock AL, Belinsky MG, Kruh GD, Dawson PA: **The organic solute transporter alpha-beta, Ost alpha-Ost beta, is essential for intestinal bile acid transport and homeostasis**. *P Natl Acad Sci USA* 2008, **105**(10):3891-3896.

80. Lee H, Zhang Y, Lee FY, Nelson SF, Gonzalez FJ, Edwards PA: **FXR regulates organic solute transporters alpha and beta in the adrenal gland, kidney, and intestine**. *J Lipid Res* 2006, **47**(1):201-214.

81. Frankenberg T, Rao A, Chen F, Haywood J, Shneider BL, Dawson PA: **Regulation of the mouse organic solute transporter alpha-beta, Ostalpha-Ostbeta, by bile acids**. *Am J Physiol Gastrointest Liver Physiol* 2006, **290**(5):G912-922.

82. Okuwaki M, Takada T, Iwayanagi Y, Koh S, Kariya Y, Fujii H, Suzuki H: **LXR alpha transactivates mouse organic solute transporter alpha and beta via IR-1 elements shared with FXR**. *Pharmaceutical research* 2007, **24**(2):390-398.

83. Khan AA, Chow ECY, Porte RJ, Pang KS, Groothuis GMM: **Expression and Regulation of the Bile Acid Transporter, OST alpha-OST beta in Rat and Human Intestine and Liver**. *Biopharm Drug Dispos* 2009, **30**(5):241-258.

84. Trauner M, Boyer JL: **Bile salt transporters: Molecular characterization, function, and regulation**. *Physiol Rev* 2003, **83**(2):633-671.

85. Renner O, Harsch S, Strohmeyer A, Schimmel S, Stange EF: **Reduced ileal expression of OSTalpha-OSTbeta in non-obese gallstone disease**. *J Lipid Res* 2008, **49**(9):2045-2054.

86. Freund JN, Domon-Dell C, Kedinger M, Duluc I: **The Cdx-1 and Cdx-2 homeobox genes in the intestine**. *Biochemistry and cell biology = Biochimie et biologie cellulaire* 1998, **76**(6):957-969.

87. Yuasa Y: **Control of gut differentiation and intestinal-type gastric carcinogenesis**. *Nature reviews Cancer* 2003, **3**(8):592-600.

88. Mallo GV, Rechreche H, Frigerio JM, Rocha D, Zweibaum A, Lacasa M, Jordan BR, Dusetti NJ, Dagorn JC, Iovanna JL: **Molecular cloning, sequencing and expression of the mRNA encoding human Cdx1 and Cdx2 homeobox. Down-regulation of Cdx1 and Cdx2 mRNA expression during colorectal carcinogenesis**. *Int J Cancer* 1997, **74**(1):35-44.

89. Subramanian V, Meyer BI, Gruss P: **Disruption of the murine homeobox gene Cdx1 affects axial skeletal identities by altering the mesodermal expression domains of Hox genes**. *Cell* 1995, **83**(4):641-653.

90. Chawengsaksophak K, James R, Hammond VE, Kontgen F, Beck F: **Homeosis and intestinal tumours in Cdx2 mutant mice**. *Nature* 1997, **386**(6620):84-87.

91. Calon A, Gross I, Lhermitte B, Martin E, Beck F, Duclos B, Kedinger M, Duluc I, Domon-Dell C, Freund JN: **Different effects of the Cdx1 and Cdx2 homeobox genes in a murine model of intestinal inflammation**. *Gut* 2007, **56**(12):1688-1695.

92. Silberg DG, Furth EE, Taylor JK, Schuck T, Chiou T, Traber PG: **CDX1 protein expression in normal, metaplastic, and neoplastic human alimentary tract epithelium**. *Gastroenterology* 1997, **113**(2):478-486.

93. Vallbohmer D, DeMeester SR, Peters JH, Oh DS, Kuramochi H, Shimizu D, Hagen JA, Danenberg KD, Danenberg PV, DeMeester TR *et al*: **Cdx-2 expression in**

**squamous and metaplastic columnar epithelia of the esophagus**. *Diseases of the Esophagus* 2006, **19**(4):260-266.

94. Hayes S, Ahmed S, Clark P: **Immunohistochemical assessment for Cdx2 expression in the Barrett metaplasia-dysplasia-adenocarcinoma sequence**. *Journal of Clinical Pathology* 2011, **64**(2):110-113.

95. Villanacci V, Rossi E, Zambelli C, Galletti A, Cestari R, Missale G, Della Casa D, Bassotti G: **COX-2, CDX2, and CDC2 immunohistochemical assessment for dysplasia-carcinoma progression in Barrett's esophagus**. *Digestive and Liver Disease* 2007, **39**(4):305-311.

96. Boudreau F, Rings EHHM, van Wering HM, Kim RK, Swain GP, Krasinski SD, Moffett J, Grand RJ, Suh ER, Traber PG: **Hepatocyte nuclear factor-1 alpha, GATA-4, and caudal related homeodomain protein Cdx2 interact functionally to modulate intestinal gene transcription - Implication for the developmental regulation of the sucrose-isomaltase gene**. *J Biol Chem* 2002, **277**(35):31909-31917.

97. Mesquita P, Jonckheere N, Almeida R, Ducourouble MP, Serpa J, Silva E, Pigny P, Silva FS, Reis C, Silberg D *et al*: **Human MUC2 mucin gene is transcriptionally regulated by Cdx homeodomain proteins in gastrointestinal carcinoma cell lines**. *Journal of Biological Chemistry* 2003, **278**(51):51549-51556.

98. Chan CWM, Wong NA, Liu Y, Bicknell D, Turley H, Hollins L, Miller CJ, Wilding JL, Bodmer WF: **Gastrointestinal differentiation marker Cytokeratin 20 is regulated by homeobox gene CDX1**. *P Natl Acad Sci USA* 2009, **106**(6):1936-1941.

99. Funakoshi S, Ezaki T, Kong JP, Guo RJ, Lynch JP: **Repression of the desmocollin 2 gene expression in human colon cancer cells is relieved by the homeodomain transcription factors Cdx1 and Cdx2**. *Mol Cancer Res* 2008, **6**(9):1478-1490.

100. Keller MS, Ezaki T, Guo RJ, Lynch JP: **Cdx1 or Cdx2 expression activates E-cadherin-mediated cell-cell adhesion and compaction in human COLO 205 cells**. *Am J Physiol-Gastr L* 2004, **287**(1):G104-G114.

101. Fang RX, Santiago NA, Olds LC, Sibley E: **The homeodomain protein Cdx2 regulates lactase gene promoter activity during enterocyte differentiation**. *Gastroenterology* 2000, **118**(1):115-127.

102. Yamamoto H, Miyamato KI, Li BL, Taketani Y, Kitano M, Inoue Y, Morita K, Pike JW, Takeda E: **The caudal-related homeodomain protein Cdx-2 regulates vitamin D receptor gene expression in the small intestine**. *Journal of Bone and Mineral Research* 1999, **14**(2):240-247.

103. Shimakura J, Terada T, Shimada Y, Katsura T, Inui K: **The transcription factor Cdx2 regulates the intestine-specific expression of human peptide transporter 1 through functional interaction with Sp1**. *Biochem Pharmacol* 2006, **71**(11):1581-1588.

104. Kakizaki F, Aoki K, Miyoshi H, Carrasco N, Aoki M, Taketo MM: **CDX transcription factors positively regulate expression of solute carrier family 5, member 8 in the colonic epithelium**. *Gastroenterology* 2010, **138**(2):627-635.

105. Takakura Y, Hinoi T, Oue N, Sasada T, Kawaguchi Y, Okajima M, Akyol A, Fearon ER, Yasui W, Ohdan H: **CDX2 Regulates Multidrug Resistance 1 Gene Expression in Malignant Intestinal Epithelium**. *Cancer Research* 2010, **70**(17):6767-6778.

106. Kazumori H, Ishihara S, Kinoshita Y: **Roles of caudal-related homeobox gene Cdx1 in oesophageal epithelial cells in Barrett's epithelium development**. *Gut* 2009, **58**(5):620-628.

107. Valente AJ, Zhou Q, Lu Z, He W, Qiang M, Ma W, Li G, Wang L, Banfi B, Steger K *et al*: **Regulation of NOX1 expression by GATA, HNF-1 alpha, and Cdx transcription factors**. *Free Radical Bio Med* 2008, **44**(3):430-443.

108. Mitchelmore C, Troelsen JT, Spodsberg N, Sjostrom H, Noren O: **Interaction between the homeodomain proteins Cdx2 and HNF1 alpha mediates expression of the lactase-phlorizin hydrolase gene**. *Biochem J* 2000, **346**:529-535.

109. Bai YQ, Yamamoto H, Akiyama Y, Tanaka H, Takizawa T, Koike M, Yagi OK, Saitoh K, Takeshita K, Iwai T *et al*: **Ectopic expression of homeodomain protein CDX2 in intestinal metaplasia and carcinomas of the stomach**. *Cancer Lett* 2002, **176**(1):47-55.

110. Almeida R, Silva E, Santos-Silva F, Silberg DG, Wang JF, De Bolos C, David L: **Expression of intestine-specific transcription factors, CDX1 and CDX2, in intestinal metaplasia and gastric carcinomas**. *J Pathol* 2003, **199**(1):36-40.

111. Mizoshita T, Inada K, Tsukamoto T, Nozaki K, Joh T, Itoh M, Yamamura Y, Ushijima T, Nakamura S, Tatematsu M: **Expression of the intestine-specific transcription factors, Cdx1 and Cdx2, correlates shift to an intestinal phenotype in gastric cancer cells**. *J Cancer Res Clin* 2004, **130**(1):29-36.

112. Eda A, Osawa H, Yanaka I, Satoh K, Mutoh H, Kihira K, Sugano K: **Expression of homeobox gene CDX2 precedes that of CDX1 during the progression of intestinal metaplasia**. *J Gastroenterol* 2002, **37**(2):94-100.

113. Mutoh H, Hakamata Y, Sato K, Eda A, Yanaka I, Honda S, Osawa H, Kaneko Y, Sugano K: **Conversion of gastric mucosa to intestinal metaplasia in Cdx2-expressing transgenic mice**. *Biochemical and biophysical research communications* 2002, **294**(2):470-479.

114. Mutoh H, Hayakawa H, Sakamoto H, Sashikawa M, Sugano K: **Transgenic Cdx2 induces endogenous Cdx1 in intestinal metaplasia of Cdx2-transgenic mouse stomach**. *Febs J* 2009, **276**(20):5821-5831.

115. Mutoh H, Sakurai S, Satoh K, Osawa H, Hakamata Y, Takeuchi T, Sugano K: **Cdx1 induced intestinal metaplasia in the transgenic mouse stomach: comparative study with Cdx2 transgenic mice**. *Gut* 2004, **53**(10):1416-1423.

116. Fan ZQ, Li JY, Dong B, Huang XF: **Expression of Cdx2 and hepatocyte antigen in gastric carcinoma: Correlation with histologic type and implications for prognosis**. *Clin Cancer Res* 2005, **11**(17):6162-6170.

117. Bonhomme C, Duluc I, Martin E, Chawengsaksophak K, Chenard MP, Kedinger M, Beck F, Freund JN, Domon-Dell C: **The Cdx2 homeobox gene has a tumour suppressor function in the distal colon in addition to a homeotic role during gut development**. *Gut* 2003, **52**(10):1465-1471.

118. Mallo GV, Soubeyran P, Lissitzky JC, Andre F, Farnarier C, Marvaldi J, Dagorn JC, Iovanna JL: **Expression of the Cdx1 and Cdx2 homeotic genes leads to reduced malignancy in colon cancer-derived cells**. *J Biol Chem* 1998, **273**(22):14030-14036.

119. Lorentz O, Cadoret A, Duluc I, Capeau J, Gespach C, Cherqui G, Freund JN: **Downregulation of the colon tumour-suppressor homeobox gene Cdx-2 by oncogenic ras**. *Oncogene* 1999, **18**(1):87-92.

120. da Costa LT, He TC, Yu J, Sparks AB, Morin PJ, Polyak K, Laken S, Vogelstein B, Kinzler KW: **CDX2 is mutated in a colorectal cancer with normal APC/beta-catenin signaling**. *Oncogene* 1999, **18**(35):5010-5014.

121. Domon-Dell C, Freund JN: **Stimulation of Cdx1 by oncogenic beta-catenin/Tcf4 in colon cancer cells; opposite effect of the CDX2 homeoprotein**. *Febs Letters* 2002, **518**(1-3):83-87.

122. Spechler SJ, Sharma P, Souza RF, Inadomi JM, Shaheen NJ: **American Gastroenterological Association technical review on the management of Barrett's esophagus**. *Gastroenterology* 2011, **140**(3):e18-52; quiz e13.

123. Spechler SJ, Goyal RK: **Barrett's esophagus**. *The New England journal of medicine* 1986, **315**(6):362-371.

124. Ronkainen J, Aro P, Storskrubb T, Johansson SE, Lind T, Bolling-Sternevald E, Vieth M, Stolte M, Talley NJ, Agreus L: **Prevalence of Barrett's esophagus in the general population: an endoscopic study**. *Gastroenterology* 2005, **129**(6):1825-1831.

125. van Soest EM, Dieleman JP, Siersema PD, Sturkenboom MC, Kuipers EJ: **Increasing incidence of Barrett's oesophagus in the general population**. *Gut* 2005, **54**(8):1062-1066.

126. Ho KY: **From GERD to Barrett's esophagus: is the pattern in Asia mirroring that in the West?** *J Gastroenterol Hepatol* 2011, **26**(5):816-824.

127. Xiong LS, Cui Y, Wang JP, Wang JH, Xue L, Hu PJ, Chen MH: **Prevalence and risk factors of Barrett's esophagus in patients undergoing endoscopy for upper gastrointestinal symptoms**. *J Dig Dis* 2010, **11**(2):83-87.

128. Amano Y, Kushiyama Y, Yuki T, Takahashi Y, Moriyama I, Fukuhara H, Ishimura N, Furuta K, Ishihara S, Adachi K *et al*: **Prevalence of and risk factors for Barrett's esophagus with intestinal predominant mucin phenotype**. *Scand J Gastroenterol* 2006, **41**(8):873-879.

129. Kim JH, Rhee PL, Lee JH, Lee H, Choi YS, Son HJ, Kim JJ, Rhee JC: **Prevalence and risk factors of Barrett's esophagus in Korea**. *J Gastroenterol Hepatol* 2007, **22**(6):908-912.

130. Park JJ, Kim JW, Kim HJ, Chung MG, Park SM, Baik GH, Nah BK, Nam SY, Seo KS, Ko BS *et al*: **The prevalence of and risk factors for Barrett's esophagus in a**

**Korean population: A nationwide multicenter prospective study**. *Journal of clinical gastroenterology* 2009, **43**(10):907-914.

131. Shaheen N, Ransohoff DF: **Gastroesophageal reflux, barrett esophagus, and esophageal cancer: scientific review**. *JAMA : the journal of the American Medical Association* 2002, **287**(15):1972-1981.
132. Thrift AP, Pandeya N, Smith KJ, Mallitt KA, Green AC, Webb PM, Whiteman DC: **Lifetime alcohol consumption and risk of Barrett's Esophagus**. *Am J Gastroenterol* 2011, **106**(7):1220-1230.
133. Anderson LA, Cantwell MM, Watson RG, Johnston BT, Murphy SJ, Ferguson HR, McGuigan J, Comber H, Reynolds JV, Murray LJ: **The association between alcohol and reflux esophagitis, Barrett's esophagus, and esophageal adenocarcinoma**. *Gastroenterology* 2009, **136**(3):799-805.
134. Steevens J, Schouten LJ, Driessen AL, Huysentruyt CJ, Keulemans YC, Goldbohm RA, van den Brandt PA: **A prospective cohort study on overweight, smoking, alcohol consumption, and risk of Barrett's esophagus**. *Cancer Epidemiol Biomarkers Prev* 2011, **20**(2):345-358.
135. Kubo A, Levin TR, Block G, Rumore GJ, Quesenberry CP, Jr., Buffler P, Corley DA: **Alcohol types and sociodemographic characteristics as risk factors for Barrett's esophagus**. *Gastroenterology* 2009, **136**(3):806-815.
136. Jacobson BC, Giovannucci EL, Fuchs CS: **Smoking and Barrett's esophagus in women who undergo upper endoscopy**. *Dig Dis Sci* 2011, **56**(6):1707-1717.
137. Smith KJ, O'Brien SM, Smithers BM, Gotley DC, Webb PM, Green AC, Whiteman DC: **Interactions among smoking, obesity, and symptoms of acid reflux in Barrett's esophagus**. *Cancer Epidemiol Biomarkers Prev* 2005, **14**(11 Pt 1):2481-2486.
138. Kubo A, Levin TR, Block G, Rumore G, Quesenberry CP, Jr., Buffler P, Corley DA: **Cigarette smoking and the risk of Barrett's esophagus**. *Cancer causes & control : CCC* 2009, **20**(3):303-311.
139. Becker L, Huang Q, Mashimo H: **Lgr5, an intestinal stem cell marker, is abnormally expressed in Barrett's esophagus and esophageal adenocarcinoma**.

*Diseases of the esophagus : official journal of the International Society for Diseases of the Esophagus / ISDE* 2010, **23**(2):168-174.

140. Souza RF, Krishnan K, Spechler SJ: **Acid, bile, and CDX: the ABCs of making Barrett's metaplasia**. *Am J Physiol Gastrointest Liver Physiol* 2008, **295**(2):G211-218.

141. Yang A, Schweitzer R, Sun D, Kaghad M, Walker N, Bronson RT, Tabin C, Sharpe A, Caput D, Crum C *et al*: **p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development**. *Nature* 1999, **398**(6729):714-718.

142. Wang X, Ouyang H, Yamamoto Y, Kumar PA, Wei TS, Dagher R, Vincent M, Lu X, Bellizzi AM, Ho KY *et al*: **Residual embryonic cells as precursors of a Barrett's-like metaplasia**. *Cell* 2011, **145**(7):1023-1035.

143. Degirolamo C, Modica S, Palasciano G, Moschetta A: **Bile acids and colon cancer: Solving the puzzle with nuclear receptors**. *Trends Mol Med* 2011, **17**(10):564-572.

144. Bernstein C, Holubec H, Bhattacharyya AK, Nguyen H, Payne CM, Zaitlin B, Bernstein H: **Carcinogenicity of deoxycholate, a secondary bile acid**. *Arch Toxicol* 2011, **85**(8):863-871.

145. Kauer WKH, Peters JH, Demeester TR, Ireland AP, Bremner CG, Hagen JA: **Mixed Reflux of Gastric and Duodenal Juices Is More Harmful to the Esophagus Than Gastric-Juice Alone - the Need for Surgical Therapy Re-Emphasized**. *Annals of Surgery* 1995, **222**(4):525-533.

146. Chen X, Oshima T, Tomita T, Fukui H, Watari J, Matsumoto T, Miwa H: **Acidic bile salts modulate the squamous epithelial barrier function by modulating tight junction proteins**. *Am J Physiol-Gastr L* 2011, **301**(2):G203-G209.

147. Nehra D, Howell P, Williams CP, Pye JK, Beynon J: **Toxic bile acids in gastro-oesophageal reflux disease: influence of gastric acidity**. *Gut* 1999, **44**(5):598-602.

148. Kaur BS, Ouatu-Lascar R, Omary MB, Triadafilopoulos G: **Bile salts induce or blunt cell proliferation in Barrett's esophagus in an acid-dependent fashion**. *Am J Physiol Gastrointest Liver Physiol* 2000, **278**(6):G1000-1009.

149. van de Winkel A, van Zoest KP, van Dekken H, Moons LM, Kuipers EJ, van der Laan LJ: **Differential expression of the nuclear receptors farnesoid X receptor (FXR)**

**and pregnane X receptor (PXR) for grading dysplasia in patients with Barrett's oesophagus**. *Histopathology* 2011, **58**(2):246-253.

150. van de Winkel A, Menke V, Capello A, Moons LM, Pot RG, van Dekken H, Siersema PD, Kusters JG, van der Laan LJ, Kuipers EJ: **Expression, localization and polymorphisms of the nuclear receptor PXR in Barrett's esophagus and esophageal adenocarcinoma**. *BMC gastroenterology* 2011, **11**:108.
151. De Gottardi A, Dumonceau JM, Bruttin F, Vonlaufen A, Morard I, Spahr L, Rubbia-Brandt L, Frossard JL, Dinjens WN, Rabinovitch PS *et al*: **Expression of the bile acid receptor FXR in Barrett's esophagus and enhancement of apoptosis by guggulsterone in vitro**. *Molecular cancer* 2006, **5**:48.
152. Capello A, Moons LM, Van de Winkel A, Siersema PD, van Dekken H, Kuipers EJ, Kusters JG: **Bile acid-stimulated expression of the farnesoid X receptor enhances the immune response in Barrett esophagus**. *Am J Gastroenterol* 2008, **103**(6):1510-1516.
153. Dvorak K, Watts GS, Ramsey L, Holubec H, Payne CM, Bernstein C, Jenkins GJ, Sampliner RE, Prasad A, Garewal HS *et al*: **Expression of bile acid transporting proteins in Barrett's esophagus and esophageal adenocarcinoma**. *Am J Gastroenterol* 2009, **104**(2):302-309.
154. Hong J, Behar J, Wands J, Resnick M, Wang LJ, DeLellis RA, Lambeth D, Souza RF, Spechler SJ, Cao W: **Role of a novel bile acid receptor TGR5 in the development of oesophageal adenocarcinoma**. *Gut* 2010, **59**(2):170-180.
155. Keswani RN, Noffsinger A, Waxman I, Bissonnette M: **Clinical use of p53 in Barrett's esophagus**. *Cancer Epidem Biomar* 2006, **15**(7):1243-1249.
156. Weston AP, Banerjee SK, Sharma P, Tran TM, Richards R, Cherian R: **p53 protein overexpression in low grade dysplasia (LGD) in Barrett's esophagus: immunohistochemical marker predictive of progression**. *Am J Gastroenterol* 2001, **96**(5):1355-1362.
157. Skacel M, Petras RE, Rybicki LA, Gramlich TL, Richter JE, Falk GW, Goldblum JR: **p53 expression in low grade dysplasia in Barrett's esophagus: correlation with interobserver agreement and disease progression**. *Am J Gastroenterol* 2002, **97**(10):2508-2513.

158. Abdel-Latif MM, O'Riordan J, Windle HJ, Carton E, Ravi N, Kelleher D, Reynolds JV: **NF-kappaB activation in esophageal adenocarcinoma: relationship to Barrett's metaplasia, survival, and response to neoadjuvant chemoradiotherapy**. *Ann Surg* 2004, **239**(4):491-500.

159. Jenkins GJ, Cronin J, Alhamdani A, Rawat N, D'Souza F, Thomas T, Eltahir Z, Griffiths AP, Baxter JN: **The bile acid deoxycholic acid has a non-linear dose response for DNA damage and possibly NF-kappaB activation in oesophageal cells, with a mechanism of action involving ROS**. *Mutagenesis* 2008, **23**(5):399-405.

160. Eda A, Osawa H, Satoh K, Yanaka I, Kihira K, Ishino Y, Mutoh H, Sugano K: **Aberrant expression of CDX2 in Barrett's epithelium and inflammatory esophageal mucosa**. *J Gastroenterol* 2003, **38**(1):14-22.

161. Wang J, Qin R, Ma Y, Wu H, Peters H, Tyska M, Shaheen NJ, Chen X: **Differential gene expression in normal esophagus and Barrett's esophagus**. *J Gastroenterol* 2009, **44**(9):897-911.

162. Kazumori H, Ishihara S, Rumi MA, Kadowaki Y, Kinoshita Y: **Bile acids directly augment caudal related homeobox gene Cdx2 expression in oesophageal keratinocytes in Barrett's epithelium**. *Gut* 2006, **55**(1):16-25.

163. Arber N, Lightdale C, Rotterdam H, Han KH, Sgambato A, Yap E, Ahsan H, Finegold J, Stevens PD, Green PH *et al*: **Increased expression of the cyclin D1 gene in Barrett's esophagus**. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* 1996, **5**(6):457-459.

164. Bani-Hani K, Martin IG, Hardie LJ, Mapstone N, Briggs JA, Forman D, Wild CP: **Prospective study of cyclin D1 overexpression in Barrett's esophagus: association with increased risk of adenocarcinoma**. *Journal of the National Cancer Institute* 2000, **92**(16):1316-1321.

165. Hong MK, Laskin WB, Herman BE, Johnston MH, Vargo JJ, Steinberg SM, Allegra CJ, Johnston PG: **Expansion of the Ki-67 proliferative compartment correlates with degree of dysplasia in Barrett's esophagus**. *Cancer* 1995, **75**(2):423-429.

166. Vaezi MF, Falk GW, Peek RM, Vicari JJ, Goldblum JR, Perez-Perez GI, Rice TW, Blaser MJ, Richter JE: **CagA-positive strains of Helicobacter pylori may protect**

**against Barrett's esophagus**. *The American journal of gastroenterology* 2000, **95**(9):2206-2211.

167. Wilson KT, Fu S, Ramanujam KS, Meltzer SJ: **Increased expression of inducible nitric oxide synthase and cyclooxygenase-2 in Barrett's esophagus and associated adenocarcinomas**. *Cancer Res* 1998, **58**(14):2929-2934.
168. Morris CD, Armstrong GR, Bigley G, Green H, Attwood SE: **Cyclooxygenase-2 expression in the Barrett's metaplasia-dysplasia-adenocarcinoma sequence**. *Am J Gastroenterol* 2001, **96**(4):990-996.
169. Shirvani VN, Ouatu-Lascar R, Kaur BS, Omary MB, Triadafilopoulos G: **Cyclooxygenase 2 expression in Barrett's esophagus and adenocarcinoma: Ex vivo induction by bile salts and acid exposure**. *Gastroenterology* 2000, **118**(3):487-496.
170. Souza RF, Shewmake K, Beer DG, Cryer B, Spechler SJ: **Selective inhibition of cyclooxygenase-2 suppresses growth and induces apoptosis in human esophageal adenocarcinoma cells**. *Cancer Res* 2000, **60**(20):5767-5772.
171. Kaur BS, Khamnehei N, Iravani M, Namburu SS, Lin O, Triadafilopoulos G: **Rofecoxib inhibits cyclooxygenase 2 expression and activity and reduces cell proliferation in Barrett's esophagus**. *Gastroenterology* 2002, **123**(1):60-67.
172. Achiwa H, Yatabe Y, Hida T, Kuroishi T, Kozaki K, Nakamura S, Ogawa M, Sugiura T, Mitsudomi T, Takahashi T: **Prognostic significance of elevated cyclooxygenase 2 expression in primary, resected lung adenocarcinomas**. *Clin Cancer Res* 1999, **5**(5):1001-1005.
173. O'Riordan JM, Abdel-latif MM, Ravi N, McNamara D, Byrne PJ, McDonald GS, Keeling PW, Kelleher D, Reynolds JV: **Proinflammatory cytokine and nuclear factor kappa-B expression along the inflammation-metaplasia-dysplasia-adenocarcinoma sequence in the esophagus**. *Am J Gastroenterol* 2005, **100**(6):1257-1264.
174. Jenkins GJ, Harries K, Doak SH, Wilmes A, Griffiths AP, Baxter JN, Parry JM: **The bile acid deoxycholic acid (DCA) at neutral pH activates NF-kappaB and induces IL-8 expression in oesophageal cells in vitro**. *Carcinogenesis* 2004, **25**(3):317-323.

175. Tselepis C, Perry I, Dawson C, Hardy R, Darnton SJ, McConkey C, Stuart RC, Wright N, Harrison R, Jankowski JA: **Tumour necrosis factor-alpha in Barrett's oesophagus: a potential novel mechanism of action**. *Oncogene* 2002, **21**(39):6071-6081.

176. Jankowski J, Murphy S, Coghill G, Grant A, Wormsley KG, Sanders DS, Kerr M, Hopwood D: **Epidermal growth factor receptors in the oesophagus**. *Gut* 1992, **33**(4):439-443.

177. Brito MJ, Filipe MI, Linehan J, Jankowski J: **Association of transforming growth factor alpha (TGFA) and its precursors with malignant change in Barrett's epithelium: biological and clinical variables**. *Int J Cancer* 1995, **60**(1):27-32.

178. Avissar NE, Toia L, Hu Y, Watson TJ, Jones C, Raymond DP, Matousek A, Peters JH: **Bile acid alone, or in combination with acid, induces CDX2 expression through activation of the epidermal growth factor receptor (EGFR)**. *Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract* 2009, **13**(2):212-222.

179. Cronin J, McAdam E, Danikas A, Tselepis C, Griffiths P, Baxter J, Thomas L, Manson J, Jenkins G: **Epidermal growth factor receptor (EGFR) is overexpressed in high-grade dysplasia and adenocarcinoma of the esophagus and may represent a biomarker of histological progression in Barrett's esophagus (BE)**. *Am J Gastroenterol* 2011, **106**(1):46-56.

180. Souza RF, Shewmake KL, Shen Y, Ramirez RD, Bullock JS, Hladik CL, Lee EL, Terada LS, Spechler SJ: **Differences in ERK activation in squamous mucosa in patients who have gastroesophageal reflux disease with and without Barrett's esophagus**. *Am J Gastroenterol* 2005, **100**(3):551-559.

181. van Baal JW, Diks SH, Wanders RJ, Rygiel AM, Milano F, Joore J, Bergman JJ, Peppelenbosch MP, Krishnadath KK: **Comparison of kinome profiles of Barrett's esophagus with normal squamous esophagus and normal gastric cardia**. *Cancer Res* 2006, **66**(24):11605-11612.

182. Sommerer F, Vieth M, Markwarth A, Rohrich K, Vomschloss S, May A, Ell C, Stolte M, Hengge UR, Wittekind C *et al*: **Mutations of BRAF and KRAS2 in the development of Barrett's adenocarcinoma**. *Oncogene* 2004, **23**(2):554-558.

183. von Rahden BH, Stein HJ, Feith M, Puhringer F, Theisen J, Siewert JR, Sarbia M: **Overexpression of TGF-beta1 in esophageal (Barrett's) adenocarcinoma is associated with advanced stage of disease and poor prognosis**. *Molecular carcinogenesis* 2006, **45**(10):786-794.

184. Luo K: **Ski and SnoN: negative regulators of TGF-beta signaling**. *Current opinion in genetics & development* 2004, **14**(1):65-70.

185. Villanacci V, Bellone G, Battaglia E, Rossi E, Carbone A, Prati A, Verna C, Niola P, Morelli A, Grassini M *et al*: **Ski/SnoN expression in the sequence metaplasia-dysplasia-adenocarcinoma of Barrett's esophagus**. *Human pathology* 2008, **39**(3):403-409.

186. Berggard T, Linse S, James P: **Methods for the detection and analysis of protein-protein interactions**. *Proteomics* 2007, **7**(16):2833-2842.

187. Phizicky EM, Fields S: **Protein-protein interactions: methods for detection and analysis**. *Microbiological reviews* 1995, **59**(1):94-123.

188. Hussain MA, Habener JF: **Glucagon gene transcription activation mediated by synergistic interactions of pax-6 and cdx-2 with the p300 co-activator**. *J Biol Chem* 1999, **274**(41):28950-28957.

189. Yarden RI, Brody LC: **Identification of proteins that interact with BRCA1 by Far-Western library screening**. *J Cell Biochem* 2001, **83**(4):521-531.

190. Defeo-Jones D, Huang PS, Jones RE, Haskell KM, Vuocolo GA, Hanobik MG, Huber HE, Oliff A: **Cloning of cDNAs for cellular proteins that bind to the retinoblastoma gene product**. *Nature* 1991, **352**(6332):251-254.

191. Blackwood EM, Eisenman RN: **Max - a Helix-Loop-Helix Zipper Protein That Forms a Sequence-Specific DNA-Binding Complex with Myc**. *Science* 1991, **251**(4998):1211-1217.

192. Fields S, Song O: **A novel genetic system to detect protein-protein interactions**. *Nature* 1989, **340**(6230):245-246.

193. Suter B, Kittanakom S, Stagljar I: **Two-hybrid technologies in proteomics research**. *Curr Opin Biotech* 2008, **19**(4):316-323.

194. Hauser R, Stellberger T, Rajagopala SV, Uetz P: **Array-based yeast two-hybrid screens: a practical guide**. *Methods Mol Biol* 2012, **812**:21-38.

195. Joung JK, Ramm EI, Pabo CO: **A bacterial two-hybrid selection system for studying protein-DNA and protein-protein interactions**. *Proc Natl Acad Sci U S A* 2000, **97**(13):7382-7387.

196. Luo Y, Batalao A, Zhou H, Zhu L: **Mammalian two-hybrid system: A complementary approach to the yeast two-hybrid system**. *Biotechniques* 1997, **22**(2):350-352.

197. Fiebitz A, Nyarsik L, Haendler B, Hu YH, Wagner F, Thamm S, Lehrach H, Janitz M, Vanhecke D: **High-throughput mammalian two-hybrid screening for protein-protein interactions using transfected cell arrays**. *Bmc Genomics* 2008, **9**.

198. von Mering C, Krause R, Snel B, Cornell M, Oliver SG, Fields S, Bork P: **Comparative assessment of large-scale data sets of protein-protein interactions**. *Nature* 2002, **417**(6887):399-403.

199. Huang H, Jedynak BM, Bader JS: **Where have all the interactions gone? Estimating the coverage of two-hybrid protein interaction maps**. *Plos Comput Biol* 2007, **3**(11):2155-2174.

200. Hall DA, Ptacek J, Snyder M: **Protein microarray technology**. *Mech Ageing Dev* 2007, **128**(1):161-167.

201. Zhu H, Bilgin M, Bangham R, Hall D, Casamayor A, Bertone P, Lan N, Jansen R, Bidlingmaier S, Houfek T *et al*: **Global analysis of protein activities using proteome chips**. *Science* 2001, **293**(5537):2101-2105.

202. Li XC, Jevnikar AM, Grant DR: **Expression of functional ICAM-1 and VCAM-1 adhesion molecules by an immortalized epithelial cell clone derived from the small intestine**. *Cellular immunology* 1997, **175**(1):58-66.

203. Eloranta JJ, Zair ZM, Hiller C, Hausler S, Stieger B, Kullak-Ublick GA: **Vitamin D3 and its nuclear receptor increase the expression and activity of the human proton-coupled folate transporter**. *Mol Pharmacol* 2009, **76**(5):1062-1071.

204. Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M, Werner T: **MatInspector and beyond: promoter analysis based on transcription factor binding sites**. *Bioinformatics* 2005, **21**(13):2933-2942.

205. Balesaria S, Pell RJ, Abbott LJ, Tasleem A, Chavele KM, Barley NF, Khair U, Simon A, Moriarty KJ, Brydon WG *et al*: **Exploring possible mechanisms for primary bile**

**acid malabsorption: evidence for different regulation of ileal bile acid transporter transcripts in chronic diarrhoea**. *Eur J Gastroenterol Hepatol* 2008, **20**(5):413-422.

206. Bergheim I, Harsch S, Mueller O, Schimmel S, Fritz P, Stange EF: **Apical sodium bile acid transporter and ileal lipid binding protein in gallstone carriers**. *J Lipid Res* 2006, **47**(1):42-50.
207. Stairs DB, Kong J, Lynch JP: **Cdx genes, inflammation, and the pathogenesis of intestinal metaplasia**. *Progress in molecular biology and translational science* 2010, **96**:231-270.
208. Wang L, Klopot A, Freund JN, Dowling LN, Krasinski SD, Fleet JC: **Control of differentiation-induced calbindin-D9k gene expression in Caco-2 cells by cdx-2 and HNF-1alpha**. *Am J Physiol Gastrointest Liver Physiol* 2004, **287**(5):G943-953.
209. Bernstein H, Bernstein C, Payne CM, Dvorak K: **Bile acids as endogenous etiologic agents in gastrointestinal cancer**. *World J Gastroentero* 2009, **15**(27):3329-3340.
210. Yang F, Huang X, Yi T, Yen Y, Moore DD, Huang W: **Spontaneous development of liver tumors in the absence of the bile acid receptor farnesoid X receptor**. *Cancer Res* 2007, **67**(3):863-867.
211. Knisely AS, Strautnieks SS, Meier Y, Stieger B, Byrne JA, Portmann BC, Bull LN, Pawlikowska L, Bilezikci B, Ozcay F *et al*: **Hepatocellular carcinoma in ten children under five years of age with bile salt export pump deficiency**. *Hepatology* 2006, **44**(2):478-486.
212. Kim I, Morimura K, Shah Y, Yang Q, Ward JM, Gonzalez FJ: **Spontaneous hepatocarcinogenesis in farnesoid X receptor-null mice**. *Carcinogenesis* 2007, **28**(5):940-946.
213. Soroka CJ, Mennone A, Hagey LR, Ballatori N, Boyer JL: **Mouse organic solute transporter alpha deficiency enhances renal excretion of bile acids and attenuates cholestasis**. *Hepatology* 2010, **51**(1):181-190.
214. Jeanmougin F, Wurtz JM, Le Douarin B, Chambon P, Losson R: **The bromodomain revisited**. *Trends in biochemical sciences* 1997, **22**(5):151-153.
215. Yamamichi N, Inada K, Furukawa C, Sakurai K, Tando T, Ishizaka A, Haraguchi T, Mizutani T, Fujishiro M, Shimomura R *et al*: **Cdx2 and the Brm-type SWI/SNF**

**complex cooperatively regulate villin expression in gastrointestinal cells**. *Exp Cell Res* 2009, **315**(10):1779-1789.

216. Lobanenkov VV, Nicolas RH, Adler VV, Paterson H, Klenova EM, Polotskaja AV, Goodwin GH: **A novel sequence-specific DNA binding protein which interacts with three regularly spaced direct repeats of the CCCTC-motif in the 5'-flanking sequence of the chicken c-myc gene**. *Oncogene* 1990, **5**(12):1743-1753.

# 8 CURRICULUM VITAE

**PERSONAL**

| | |
|---|---|
| Name: | Li Ma, Ms.c. |
| Citizenship: | China |
| Date of Birth: | October 26, 1983 |
| Address: | Greifenseestrasse 33<br>8050, Zurich, Switzerland |
| Phone: | 0041-77-463-5256 |
| Email: | malivsmali@gmail.com |


**EDUCATION AND QUALIFICATIONS**

2008-2012 University of Zurich
PhD candidate
University Hospital Zurich, Division of Clinical Pharmacology and Toxicology
Thesis entitled: Regulation of the intestinal bile acid transporter genes by the transcription factors CDX1 and CDX2"
Technical skills: Cell culture, molecular cloning, western blotting, luciferase reporter assays, electromobility shift assays, chromatin immunoprecipitation assays

2005-2007 Oriel College, University of Oxford and Department of Clinical Medicine
M.Sc. Immunology
Thesis entitled: Antigen specific T cell analysis in HIV/MTB co-infected women and infants
Research skills: Scientific presentation and proficient in Microsoft Word, Excel and Prism (statistical analysis software)
Technical skills: ELISPOT assay, PBMC separation from blood, FACS.
Attended the 4th UK Meeting on the Biology and Pathology of Hepatitis C Virus

2002-2005 Queen Mary and Westfield College, University of London
B.Sc. Molecular Biology
Final year research project on RNA silencing in *Schizosaccharomyces pombe*
Technical skills: Basic techniques in molecular biology

2000-2002 Duff Miller College, 59 Queen's Gate, London, SW7 5JP, UK
A Level Mathematics
A Level Biology
A Level Chemistry
Overall band of 7.5 in the IELTS (International English Language Testing

System) exam in May 2002

**TEACHING EXPERIENCE**

2008-2011 Membrane transport, Respiration, Ergometry, Vision. Teaching assistant for 1st and 2nd year medical students at practical courses. Institute of Physiology, University of Zurich

**PRESENTATION**

2008 Young investigators meeting. Konolfingen, entitled "Regulation of the genes encoding the intestinal bile acid transporters ASBT and OSTα by the CDX transcription factors"

2009 Pharma Poster Day, Zurich, entitled "Regulation of the *ASBT* gene by transcription factor CDX1"

2011 Digestive disease week (DDW), Chicago, Poster presentation entitled "Regulation of the gene encoding the intestinal bile acid transporter ASBT by CDX transcription factors"

**OTHER SKILLS**

- Chinese (Native), English (Proficient in writing and speaking)
- Microsoft Office: Word, Excel, PowerPoint, Outlook.GraphPad.

**PUBLICATIONS**

1 **Li Ma,** Moritz Jüttner, Gerd A. Kullak-Ublick, and Jyrki J. Eloranta, Regulation of the gene encoding the intestinal bile acid transporter ASBT by the caudal-type homeobox proteins CDX1 and CDX2. *Am J Physiol Gastrointest Liver Physiol January 2012 302:(1) G123-G133*

# 9 ACKNOWLEDGMENTS

First of all, I would like to acknowledge Professor Bruno Stieger whom I met in 2005 for introducing me to the Department of Pharmacology and Toxicology at University Hospital of Zurich, which later led to a PhD position. I would also like to take this opportunity to thank my former research mentors Professor Conrad Lichtenstein and Professor Paul Klenerman for their guidance.

My deepest gratitude goes to Professor Gerd Kullak for offering me this wonderful opportunity to undertake the PhD thesis in his lab group and the beautiful city of Zurich, and his continuous support, mentorship and guidance in the past four years. I have worked closely with PD. Dr. Jyrki Eloranta, from whom I have learnt the ins and out of many techniques, and he has always been approachable. I am grateful for your patience and generous help during the manuscript and thesis writing. I thank you both for the personal and professional development in the Department. It would have not been possible to complete this thesis without both of you. I want to express my sincere thanks to my thesis committee members: Prof. Dr. Carsten Wagner and Prof. Dr. Béatrice Desvergne for their good advice and support in the PhD committee meetings.

I am grateful to the colleagues in China: Dr. Xiangjun Jiang, Dr. Zhanju Liu, Dr. Jun Wan, Dr. Haifeng Liu, for the donation of Barrett's esophagus tissue samples for the CDX/ASBT study. Without these materials, it would be impossible to publish an interesting translational study.

Many thanks go to our past and current division secretaries: Ms Kerstin Hartung and Ms Rose Bosshard for all the paper work and organization of many memorable division outdoor activities and events.

I want to express my many thanks to my fellow colleagues for their support, it has always been great fun to work in the lab: Moritz, Ragam, Carlos, Christelle, Lia, Christian F., Katrin, and Zhibo. Especially Christian H. for his technical advice; he has always been so good at

explaining complicated methodologies. Former colleagues, Kerstin, Christoph, and Christa, are acknowledged for helpful discussions.

Many thanks to my dearest friends for their support and making the most enjoyable years of my life in Zurich. This has made my time enriched with many other things.

Last but not the very least, I am greatly indebted to my families, my parents and my sister for their unconditional love, encouragement, who have supported me through all the way, their understanding and patience.

# 10 APPENDIX

**Table 1** Sequences of oligonucleotides used for cloning, SDM, EMSAs, and ChIP assays. Where applicable, restriction sites introduced are underlined and the corresponding enzymes used are given in parentheses. Only the top strands are shown for oligonucleotides used in EMSAs. *CDXRE* consensus motifs are indicated in bold and introduced mutations are in italics.

| | **Sequence (5'-3')** |
|---|---|
| **Cloning oligonucleotides** | |
| *hASBT* -99/+525 forward | GAGCTCGGAAATTGGATGGAGATCTGGGTC (Sac I) |
| *hASBT* -99/+525 reverse | AAGCTTCTGGCTCTGCTGCTGGTTGAGTT (Hind III) |
| *mAsbt* -1810/+3 forward | GAGGTAAGGTACCAGAGATCAATCAGACCGATG (Kpn I) |
| *mAsbt* -1810/+3 reverse | ACAGAAGCACCAGATCTGTCTCTACTGCTAGCTG (Bgl II) |
| *rAsbt* -2975/+147 forward | GGTACCAACATTCTTAATCAAAGTCTATATGAG (Kpn I) |
| *rAsbt* -2975/+147 reverse | GCTAGCGCAGAAAGTTGCATTTGGG (Nhe I) |
| **SDM oligonucleiotides** | |
| *CDXRE*_2 | GTTGAATACTGAATTAGAATTTGTCCTACAAATTGTG |
| *CDXRE*_3 | GGAATATTCAATGTGATTATTACTCTGTGTCCTAACTC |
| *CDXRE*_4 | CTGGGTTCTGAACTCCTGAAAACAGGACAATCTCTTC |
| *CDXRE*_8 | CCATCTCTGAAGTACTCTGTTACTATGACAACGC |
| *CDXRE*_9 | CATATACGTGATGGACACTACAAAGCAATTCC |
| *CDXRE*_10 | GGCTCTTGTGATATGGCATGGTTCATTATC |
| *CDXRE*_11 | GCCACTGAATGATTAATCGTAACTCTCTGTC |
| *CDXRE*_12 | CTAGCGTGATTGTTTGTGATTACTTCCCTAAGGTGGCTTTC |
| **EMSA oligonucleotides** | |
| *CDXRE*_1_top | AGCTTGAATA**ATAAA**TTAGAATT |
| *CDXRE*_2_top | AGCTATTCAAT**TTTAT**TATTACT |
| *CDXRE*_3_top | AGCTCTGGGTT**ATAAA**CTCCTGA |
| *CDXRE*_4_top | AGCTATTTCAA**TTTAT**GACTCCAA |
| *CDXRE*_5_top | AGCTCATCT**ATAAA**GTACTCTG |
| *CDXRE*_6_top | AGCTCATATAC**TTTAT**GGACACT |
| *CDXRE*_1_top_mut | AGCTTGAATA***CTGAA***CTTGAATT |

| | |
|---|---|
| *CDXRE*_2_top_mut | AGCTATTCAAT***GT******G*****AT**TATTACT |
| *CDXRE*_3_top_mut | AGCTCTGGGTT***C*****T*****G*****AA**CTCCTGA |
| *CDXRE*_4_top_mut | AGCTATTTCAA***GT******G*****AT**GACTCCAA |
| *CDXRE*_5_top_mut | AGCTTCCATCT***C*****T*****G*****AA**GTACTCT |
| *CDXRE*_6_top_mut | AGCTCATATAC***GT******G*****AT**GGACACT |
| *CDX*_ consensus | AGCTAATGATAATTTTATGGATAT |

**ChIP oligonucleotides**

| | |
|---|---|
| *hASBT*_forward -736 | GGGAGTGCTAATCCTACTTAATTTAAGATG |
| *hASBT*_reverse -154 | CTGTATCAGTGTTTCCTGGAATTGCTTTGTAG |
| *hASBT*_forward intron 1 | CACCTTGGAGTAATCCTTCCAGAAGCCT |
| *hASBT*_reverse intron 1 | GAGATCAACAGCAATTATGCAACTCCCTGT |

# 11 DECLARATION

Experiment Fig 4.3 (F&G) was performed by Moritz Jüttner